Dark Fermentative Biohydrogen Production using South African Agricultural, Municipal and Industrial Solid Biowaste Materials

Patrick Thabang Sekoai

A dissertation submitted to the Faculty of Engineering and the Built Environment, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of Doctor of Philosophy in Engineering

Supervisor: Professor M.O. Daramola

October 2017

Declaration

I declare that this dissertation is my own unaided work. It is being submitted for the degree of Doctor of Philosophy in Engineering to the University of the Witwatersrand, Johannesburg, South Africa. It has not been submitted before for any degree or examination to any other University.

..……...

(Signature of Candidate)

...……day of……2017

(Day) (Month) (Year)

Abstract

The dwindling fossil reserves coupled with environmental pollution necessitate the search for clean and sustainable energy resources. Biohydrogen is emerging as a suitable alternative to fossil fuels and has received considerable attention in recent years due to its economic, social, and environmental benefits. However, the industrial application of biohydrogen has been hindered by low yield. Therefore, development of novel techniques to enhance the yield is of immense importance towards large-scale production of biohydrogen.

Thus, this research effort explored various options to enhance the yield of biohydrogen during dark fermentation process. Some options explored included (i) the utilization of feedstocks from the agricultural, industrial and municipal sectors, (ii) parametric optimization of biohydrogen production, (iii) investigation of biohydrogen production using metal ions and nitrogen gas sparging, and (iv) assessing the feasibility of biohydrogen scale-up study to pave the way for pilot-scale development. Solid biowaste feedstocks consisting of apple, bread, brewery residue, cabbage, corn-cob, mango, mealie-pap, pear, potato, and sugarcane were investigated for dark fermentative biohydrogen production using anaerobic mixed sludge. The experimental results showed that substrates which are rich in carbohydrates are suitable for dark fermentative biohydrogen-producing bacteria. Consequently, a maximum biohydrogen fraction of 43.98, 40.32 and 38.12% with a corresponding cumulative biohydrogen yield of 278.36, 238.32 and 215.69 mL H_2/g total volatile solids (TVS) was obtained using potato, cabbage, and brewery wastes, respectively. Based on these results, potato waste was chosen as a suitable substrate for subsequent biohydrogen production studies.

Parametric optimization was carried out on biohydrogen production via dark fermentation using potato waste as the substrate. Effects of operating variables such as pH, temperature,

fermentation time, and substrate concentration were investigated via response surface methodology (RSM) approach using a two-level-four factor (2^4) central composite design (CCD). The obtained predictive model (statistical model) was used to explain the main and interaction effects of the considered variables on biohydrogen production. In addition, the model was employed in the optimization of the operating conditions. Consequently, a second-order polynomial regression with a coefficient of determination (R^2) of 0.99 was obtained and used in the explanation and optimization of operating variables. The optimum operating conditions for biohydrogen production were 39.56 g/L, 5.56, 37.87 ^oC and 82.58 h for potato waste concentration, pH, temperature and fermentation time, respectively, with a corresponding biohydrogen yield of 68.54 mL H_2/g TVS. These results were then validated experimentally and a high biohydrogen yield of 79.43 mL H_2/g TVS indicating a 15.9% increase was obtained. Furthermore, the optimized fermentation conditions were applied in the scale-up study of biohydrogen production that employed anaerobic mixed bacteria (sludge) which was immobilized in calcium alginate beads. A biohydrogen fraction of 56.38% with a concomitant yield of 298.11 mL H_2/g TVS was achieved from the scale-up study.

The research also investigated the influence of metal ions (Fe^{2+}, Ca^{2+}, Mg^{2+} and Ni^{2+}) on biohydrogen production from suspended and immobilized cells of anaerobic mixed sludge using the established optimal operating conditions. A maximum biohydrogen fraction of 45.21% and a corresponding yield of 292.8 mL H_2/g TVS was achieved in fermentation using Fe^{2+} (1000 mg/L) and immobilized cells. The yield was 1.3 times higher than that of suspended cultures. The effect of nitrogen gas sparging on biohydrogen conversion efficiency (via suspended and immobilized cells) was studied as well. Cell immobilization and nitrogen gas sparging were effective for biohydrogen production enhancement. A maximum biohydrogen fraction of 56.98% corresponding to a biohydrogen yield of 294.83 mL H_2/g

TVS was obtained in a batch process using nitrogen gas sparging with immobilized cultures. The yield was 1.8 and 2.5 times higher than that of nitrogen gas sparged and non-sparged suspended cell system, respectively.

Understanding the functional role of microorganisms that actively participate in dark fermentation process could provide in-depth information for the metabolic enhancement of biohydrogen-producing pathways. Therefore, the microbial composition in the fermentation medium of the optimal substrate (potato waste) was examined using PCR-based 16S rRNA approach. Microbial inventory analysis confirmed the presence of *Clostridium* species which are the dominant biohydrogen-producing bacteria.

The results obtained from this research demonstrated the potential of producing biohydrogen using South African solid biowaste effluents. These feedstocks are advantageous in biohydrogen production because they are highly accessible, rich in nutritional content, and cause huge environmental concerns. Furthermore, optimization techniques using these feedstocks will play a pivotal role towards large-scale production of biohydrogen by increasing throughput and reducing the substrate costs which accounts for approximately 60% of the overall costs. The findings from this research also provide a solid basis for further scale-up and techno-economic studies. Such studies are necessary to evaluate the competitiveness of this technology with the traditional processes of hydrogen production. In summary, the findings from this research effort have been communicated to researchers in the area of biohydrogen process development in the form of peer-reviewed international scientific publications and conference proceedings, and could provide a platform for developing an economic biohydrogen scaled-up process.

Acknowledgements

First and Foremost, I would like to thank the Lord Almighty for the countless blessings in my life and giving me perseverance during the course of this research. The journey towards the completion of this doctoral degree was a tumultuous one, but I could not have reached so far without his love and guidance.

I am highly indebted to my supervisor, Professor Michael Daramola for granting me an opportunity to pursue my doctorate under his supervision. His valuable insights, kindness, and persevering spirit gave me strength to continue with this endeavour. I will forever cherish him and may God continue to bless him.

I would also like to extend my deepest gratitude to Professor Esther Akinlabi and Mr. Rueben Shuma from the University of Johannesburg (Department of Mechanical Engineering, Kingsway campus) for allowing me to use their facilities. This research would not have been possible without their contribution. I cannot forget Mr. Motlatsi Phali and Mr. Doctor Mbense for their assistance with research equipment during the course of this research.

I extend my appreciation to The National Research Foundation (NRF) and Postgraduate Merit Scholarship (Wits PMA) for financial support during my postgraduate studies.

I also thank my research colleagues from Richard Ward Building; Kevin Yoro, Lawrence Obidike, Siphesihle Mbatha, Aliki Makumba, Ayotunde Awosusi, Michael Bodunrin, Damilola McGregor, Wumi Sadare, Demmy Fayemiwo, Olawale Oloye, and Dr. Augustine Ayeni for their friendship, support and making my time valuable at Wits.

Finally, I thank my only surviving family member, my Aunt (Anna Bongekile Mndaweni) for her continued love and support.

Dedication

This dissertation is dedicated to my late mother **Agnes Sekoai**, May 1968−June 2003.

Thank you for teaching me the importance of hard work, persistence, and purposeful life. You are dearly missed, rest in peace.

Publications from the study

A few publications which emanated from this study are highlighted below. This thesis is a careful compilation of these publications.

Peer-reviewed Journal Articles:

1. **Sekoai, P.T.,** Awosusi, A.A., Yoro, K.O., Singo, M., Oloye, O., Ayeni, A.O., Bodunrin, M., Daramola, M.O. (2017). Microbial cell immobilization in biohydrogen production−A short overview. *Critical Reviews in Biotechnology, 1-15*.

2. **Sekoai, P.T.,** Daramola, M.O. (2017). The Potential of Dark fermentative Bio-hydrogen Production from Biowaste Effluents in South Africa−Review. *International Journal of Renewable Energy Research 7(1), 359-378.*

3. **Sekoai, P.T.** (2016). Modelling and Optimization of Operational Setpoint Parameters for Maximum Fermentative Biohydrogen Production Using Box-Behnken Design. *Fermentation 2(15), 1-11*.

4. **Sekoai, P.T.,** Yoro, K.O., Daramola, M.O. (2016). Batch Fermentative Biohydrogen Production Process Using Immobilized Anaerobic Sludge from Organic Solid Waste. *Environments 3(38), 1-10.*

5. **Sekoai, P.T.,** Daramola, M.O. (2015). Biohydrogen production as a potential energy fuel in South Africa−Mini Review. *Biofuel Research Journal 6, 223-226.*

6. **Sekoai, P.T.,** Yoro, K.O. (2016). Biofuel Development Initiatives in Sub-Saharan Africa: Opportunities and Challenges−Review. *Climate 4(33), 1-13.*

7. Yoro, K.O., **Sekoai, P.T.** (2016). The Potential of CO_2 Capture and Storage Technology in South Africa's Coal-Fired Thermal Power Plants − Review. *Environments 3(24), 1-20.*

8. **Sekoai, P.T.,** Ayeni, A.O., Daramola, M.O. (2017). Parametric optimization of biohydrogen production from potato waste and scale-up study using immobilized anaerobic sludge. *Waste and Biomass Valorization (under review).*

9. **Sekoai, P.T.,** Daramola, M.O. (2017). Effect of metal ions on dark fermentative biohydrogen production using suspended and immobilized cells of mixed bacteria. *Chemical Engineering Communications (under review)*.

Peer-reviewed Conference Proceedings/Oral Presentations:

1. **Sekoai, P.T.,** Daramola, M.O. Dark fermentative biohydrogen production using South African biowaste effluents. Third National Conference on Global Change. Southern Sun Hotel, Elangeni, Durban, South Africa, 5 – 8 December 2016. **Oral Presentation.**

2. **Sekoai, P.T.,** Shuma, R.M., Akinlabi, E.T., Daramola, M.O. Biogenic biohydrogen production using South African solid biowaste effluents. The 2nd International Conference on Energy, Environment and Climate (ICEECC). Le Meridien Hotel, Pointe aux Piments, Mauritius, 5 – 7 July 2017. **Oral Presentation.**

3. Makgaba, C.P., **Sekoai, P.T.,** Daramola, M.O. Waste to Energy: Conversion of animal fat to biodiesel over a solid Hydroxy Sodalite (HS) catalyst. The 2nd International Conference on Energy, Environment and Climate (ICEECC). Le Meridien Hotel, Pointe aux Piments, Mauritius, 5 – 7 July 2017. **Oral Presentation.**

Table of Contents

Lists of Figures

Lists of Tables

Abbreviations

APHA	American Public Health Association
ATP	Adenosine Triphosphate
BOD	Biochemical Oxygen Demand
CCD	Central Composite Design
CH_4	Methane
CO_2	Carbon dioxide
COD	Chemical Oxygen Demand
DNA	Deoxyribonucleic Acid
GC	Gas Chromatography
h	hour
H_2	Hydrogen
L	Litre
min	minutes
mm	millimetre
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
rpm	revolutions per minute
RSM	Response Surface Methodology
TVS	Total Volatile Solids
VFAs	Volatile Fatty Acids

Chapter 1–Introduction

The motivation for this study and the research objectives are clearly defined in this chapter. The social, economic and environmental benefits of the research effort are also highlighted.

1.1 Background and Motivation

During the twentieth century, majority of research focused on the exploitation of fossil fuels such as crude oil, coal, and natural gas for energy supply because they were abundant and inexpensive (Bender, 2000; Demirbas, 2006). However, these energy reserves are no longer sustainable and pose huge environmental concerns. The burning of fossil fuels has drastically increased the levels of carbon dioxide, a greenhouse gas that is directly associated with global warming (Naik et al., 2015). It has been predicted that the amounts of atmospheric CO_2 might reach 560 ppm in 2035 with a temperature rise that could exceed 5 °C (Stern, 2008). The effects of greenhouse gas emissions are catastrophic; they include heat stress, floods, droughts, and health problems due to harsh weather patterns (Cooper et al., 2008; Rosenzweig et al., 2002). Furthermore, fossil fuels are geographically unevenly distributed and are being exhausted (Bentley, 2002). This has led to unstable energy prices. Another looming crisis is that the world population is increasing at an exponential rate; thus the existing energy reserves will not cope with increasing energy demands (Asif and Muneer, 2007). It is also predicted that by 2050, the energy demands will exceed supply (Holmes and Jones, 2003).

In recent years, there has been an upsurge of interest in the intensification of clean and sustainable energy resources in order to mitigate these challenges (Lennartsson et al., 2014; Kumar et al., 2015; Sekoai and Yoro, 2016). Hydrogen is considered as one of the most suitable energy resource due to its non-pollution characteristics (produces only water upon combustion), it has a high energy yield (122 kJ/g) that is 2.75 times higher than that of fossil

fuels, it can be generated using various methods including inexpensive processes, and is used in many industrial applications (Sekoai and Daramola, 2015). It is commercially produced using thermo-chemical processes like steam reforming which are energy intensive and contribute to carbon emissions (Saratale et al., 2008). Therefore, an emphasis is being put on biotechnological processes to generate cleaner hydrogen energy. Biotechnological hydrogen-producing methods include: (i) biophotolysis uses phototrophic microorganisms such as cyanobacteria and split water molecules into hydrogen and oxygen, (ii) photo-fermentation employs phototrophic microorganisms to produce hydrogen under an illumination source, and (iii) the dark fermentation process uses heterotrophic microorganisms to produce hydrogen. Hydrogen production via dark fermentation is more beneficial because this process employs diverse feedstocks including waste materials, produces hydrogen at ambient temperature and pressure, uses obligate and facultative anaerobes that are found in various habitats, reduces the levels of contamination due to its ability to use diverse microbial communities, offers a simple process, and has a potential for large-scale production (Hallenbeck and Ghosh, 2009).

Despite its merits, the process of dark fermentation is affected by low yields which hinder its commercialization. The experimental yields are lower than the theoretical yields due to the presence of biohydrogen-inhibiting reactions that lowers the overall conversion efficiency (Das and Veziroglu, 2008). Hitherto, the highest experimental yield reported in literature is 2.3 mol H_2 mol^{-1} glucose and is about 57% of the theoretical yield (Wong et al., 2014). Theoretically, 4 mol H_2 mol^{-1} glucose is produced from the acetic acid reaction, whereas 2 mol H_2 mol^{-1} glucose is generated from the butyric acid reaction as shown in Equations 1.1 and 1.2, respectively:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (1.1)$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (1.2)$$

Most biohydrogen optimization approaches documented in literature use monomeric sugars such as glucose (Karthic et al., 2012), sucrose (Sun et al., 2010), galactose (Xia et al., 2016), and xylose (Chaganti et al., 2012). These substrates are expensive and will therefore escalate the process costs at large-scale. In order to make the process economically viable, biohydrogen should be produced from feedstocks that are easily accessible, cheap, rich in nutritional content, and considered waste materials. Furthermore, enhancement strategies using these feedstocks will increase throughput, reduce the process costs, and can provide scalable fermentation data.

1.2 Problem statement

Even though Africa produces about 10 PJ to 525 PJ (PJ-Pentajoule: 10^{15}) of biomass residues each year (Stecher et al., 2013), research regarding the utilization of these waste materials for alternative energy production is scarce in literature. Thus, this impedes initiatives for development of clean and sustainable energy production within the continent. Dark fermentative biohydrogen production has the potential to replace the existing processes relying heavily on hydrocarbon fuels. However, its large-scale production has been hampered by low conversion yields on substrates (Das and Veziroglu, 2001). In South Africa, agricultural, industrial and municipal waste products are seen as cheap feedstock for biohydrogen process development due to their nutritional composition, environmental consequences, and accessibility. Furthermore, it has been shown that these biomass materials will increase by approximately 11 million tons per year over the next decades due to high level of infrastructure development occurring in most cities around the country (Department of Environmental Affairs, 2014). Therefore, further studies on the production of biohydrogen and its optimization using these feedstocks will contribute enormously towards biohydrogen production process advancement.

1.3 Research Hypotheses and Questions

In this research, it was expected that nutrient-rich feedstocks might generate maximum biohydrogen yield due to their biodegradable nature and nutritional content. It was also expected that biohydrogen production might be influenced by its operating conditions. In addition, it was anticipated that the biohydrogen yield might be enhanced by the metal ions and nitrogen gas sparging during dark fermentation process. To prove these hypotheses, the following questions were investigated during the course of this research.

i. Which of the South African solid biowaste effluents could be used to achieve a high biohydrogen production yield?

ii. What is the genome of microorganisms that actively participate in dark fermentative biohydrogen production using South African solid biowaste effluents?

iii. What will be the effect of operating variables on biohydrogen production yield during dark fermentation process using South African solid biowaste effluents?

iv. What will be the effect of metal ions on biohydrogen production yield during dark fermentation process using South African solid biowaste effluents?

v. What will be the effect of nitrogen gas sparging on biohydrogen production yield during dark fermentation process using South African solid biowaste effluents?

1.4 Research Aim and Objectives

The aim of this research is to produce and maximize biohydrogen production yield using agricultural, municipal and industrial solid biowaste effluents from South Africa. To achieve this goal, the following specific objectives were carried out:

i. Screening of South African agricultural, municipal, and industrial solid biowaste materials for dark fermentative biohydrogen production.

ii. Identification of biohydrogen-producing microorganisms in the fermentation medium of the optimal substrate obtained in (i), using PCR-based 16S rRNA approach.

iii. Investigation of operating variables of temperature, pH, fermentation time, and substrate concentration on biohydrogen yield via response surface methodology (RSM) approach using the optimal substrate identified in (i). And a biohydrogen scale-up study using the established optimized conditions and immobilized bacteria as the inoculum.

iv. Investigation of the effect of Fe^{2+}, Ca^{2+}, Mg^{2+} and Ni^{2+} ions on biohydrogen production using suspended and immobilized cells of anaerobic mixed sludge.

v. Investigation of the effect of nitrogen gas sparging on biohydrogen production using suspended and immobilized cells of anaerobic mixed sludge.

1.5 Thesis layout

Chapter 1:

This chapter presents a general background and motivation of this research. It also describes the aims and objectives of this work.

Chapter 2:

This chapter provides an outlook on the energy sector in South Africa and evaluates the various energy production methods that are used in the country. It highlights the drawbacks of energy derived from fossil fuels. It examines the country's efforts towards the intensification of clean and sustainable energy like dark fermentation process. Furthermore, it elucidates the potential of agricultural, municipal, and industrial waste as feedstocks for dark fermentative biohydrogen production in South Africa. Two review articles published in "*International Journal of Renewable Energy Research*" and "*Biofuel Research Journal*" emanated from this chapter. Copies of these papers are provided in the Appendix A of this thesis.

Chapter 3:

This chapter reports the results of the screening of South African solid biowaste effluents for the dark fermentative biohydrogen production. Selected biowaste materials from the agricultural, industrial and municipal sector were investigated for dark fermentative biohydrogen production using anaerobic mixed sludge. These substrates were distinguished based on their biohydrogen production yields. The study also seeks to understand the microorganisms that play a role during dark fermentation process. The results obtained from this study have been published in the Proceedings of the *2nd International Conference on Energy, Environment and Climate Change (ICEECC)*, which was held in Mauritius on the 5th - 7th July 2017 (see Appendix A).

Chapter 4:

This chapter focuses on the parametric optimization of biohydrogen production via response surface methodology (RSM) approach using a central composite design (CCD) on the design of experiments (DOE). The optimized operating conditions were used in biohydrogen-scale up study that employed immobilized bacteria. Results of the investigation were published in "*Environments*", an open access international journal. In addition, a review article which explores the potential of cell immobilization in biohydrogen production was published in "*Critical Reviews in Biotechnology*". Results of the detailed investigation as documented in this chapter are currently being reviewed at "*Waste and Biomass Valorization*". These papers are included in the Appendix A of this thesis.

Chapter 5:

This chapter presents the results of the investigation of the effect of metal ions (F^{2+}, Ca^{2+}, Mg^{2+} and Ni^{2+}) on biohydrogen production using suspended and immobilized cells. The findings from this work have been compiled in a manuscript that is currently under review at "*Chemical Engineering Communications*" (see Appendix A).

Chapter 6:

This chapter is dedicated to understanding the effect of nitrogen gas sparging on biohydrogen production using suspended and immobilized cells.

Chapter 7:

This chapter provides conclusions and recommendations from this study.

1.6 Contribution to knowledge

This research provides information about the use of South African solid biowaste effluents in dark fermentative biohydrogen production. The results obtained from this research also provide in-depth knowledge on the key operating variables (pH, temperature, fermentation time, and substrate concentration) that affect the biohydrogen production yield. In addition, this study established the relative advantages of employing immobilized bacteria in biohydrogen production over the non-immobilized cultures. The dissertation also provides information of the scale-up study that could pave the way for commercialization of the process. To provide quick access to the information documented in this thesis, results of this study have been communicated to the scientific community through peer-reviewed journal articles and contributions to conferences (see Appendix A for copies of these contributions).

References

Asif, M., and Muneer, T. (2007). Energy supply, its demand and security issues for developed and emerging economies. Renewable and Sustainable Energy Reviews 11, 1388-1413.

Bender, M. (2000). Potential of biomass in the production of synthetic organics. Resource Conservation and Recycling 30, 49-58.

Bentley, R.W. (2002). Global oil & gas depletion: an overview. Energy Policy 30, 189-205.

Chaganti, S.R., Kim, D.K., Lalman, J.A., and Shewa, W.A. (2012). Statistical optimization of factors affecting biohydrogen production from xylose fermentation using inhibited mixed anaerobic cultures. International Journal of Hydrogen Energy 37, 11710-11718.

Cooper, P., Dimes, J., Rao, K., Shapiro, B., Shapiro, B., Shiferaw, B., and Twomlow, S. (2008). Coping better with current climatic variability in the rain-fed farming systems of sub-Saharan Africa: an essential first step in adapting to future climate change? Agriculture, Ecosystems and Environment 126, 24-35.

Das, D., and Veziroglu, T.N. (2001). Hydrogen production by biological processes: a survey of literature. International Journal of Hydrogen Energy 26, 13-28.

Demirbas, M.F. (2006). Current technologies for biomass processes into chemical and fuels. Energy Sour Part A 28, 1181-1188.

Department of Environmental Affairs, (2014). National waste information. Baseline Report, Pretoria.

Hallenbeck, P.C., and Ghosh, D. (2009). Advances in fermentative biohydrogen production: the way forward? Trends in Biotechnology 27, 287-297.

Holmes, B., and Jones, N. (2003). Brace yourself for the end of cheap oil. New Scientist 179, 9-11.

Karthic, P., Joseph, S., and Arun, N. (2012). Optimization of Process Variables for Biohydrogen Production from Glucose by *Enterobacter aerogenes*. Journal of Microbial & Biochemical Technology 1, 1-5.

Kumar, G., Bakonyi, P., Periyasamy, S., Kim, S.H., Nemestóthy, N., and Bélafi-Bakó, K. (2015). Lignocellulose biohydrogen: Practical challenges and recent progress. Renewable and Sustainable Energy Reviews 44, 728-737.

Lennartsson, P.R., Erlandsson, P., and Taherzadeh, M.J. (2014). Integration of the first- and second-generation bioethanol processes and the importance of by-products. Bioresource Technology 165, 3-8.

Naik, N.S., Goud, V.V., Rout, P.K., and Dalai, A.K. (2015). Production of first and second generation biofuels: A comprehensive review. Renewable and Sustainable Energy Reviews 14, 578-597.

Rosenzweig, C., Tubiello, F.N., Goldberg, R., Mills, E., and Bloomfield, J. (2002). Increased crop damage in the US from excess precipitation under climate change. Global Environmental Change 12, 197-202.

Saratale, G.D., Chen, S.D., Lo, Y.C., Saratale, R.G., and Chang, S. C. (2008). Outlook of biohydrogen production from lignocellosic feedstock using dark fermentation-a review. Science 345, 1326-1330.

Sekoai, P.T., and Daramola, M.O. (2015). Biohydrogen production as a potential energy fuel in South Africa. Biofuel Research Journal 6, 223-226.

Sekoai, P.T., and Yoro, K.O., (2016). Biofuel development Initiatives in Sub-Saharan Africa: Opportunities and Challenges. Climate 4, 1-13.

Stecher, K., Browski, A., and Thran, D. (2013). Biomass potential in Africa. International

Renewable energy agency. Available at: https://www.irena.org/DocumentDownloads/Publications/IRENA-DBFZ_Biomass%20Potential%20in%20Africa.pdf (Accessed 8 March 2016).

Stern, N. (2008). The economics of climate change. American Economic Review 98, 1-37.

Sun, Q., Xiao, W., Xi, D., Shi, J., Yan, X., and Zhou, Z. (2010). Statistical optimization of biohydrogen production from sucrose by a co-culture of *Clostridium acidisoli* and *Rhodobacter sphaeroides*. International Journal of Hydrogen Energy 35, 4076-4084.

Wong, Y.M., Wu, T.Y., and Juan, J.C. (2014). A review of sustainable hydrogen production using seed sludge via dark fermentation. Renewable and Sustainable Energy Reviews 34, 471-482.

Xia, A.O., Jacob, A., Hermann, C., and Murphy, J.D. (2016). Fermentative bio-hydrogen production from galactose. Energy 96, 346-354.

Chapter 2–Literature Review

This chapter provides an overview of South Africa's energy sector. It examines the various energy resources that are used in the country and discusses the challenges caused by energy derived from fossil fuels. It explores the country's efforts towards the intensification of clean and sustainable energy resources like dark fermentative biohydrogen production. Furthermore, it explains the technical barriers faced by dark fermentation process and concludes by proposing different strategies that could be used to accelerate its large-scale production from biowaste feedstocks.

2.1 Introduction

The reliance on fossil fuels has resulted in severe challenges of greenhouse gas emissions, environmental concerns, and escalating energy demands (Davila-Vazquez et al., 2008). The United Nations predicted a global population of 6.8 billion in 2009 and expects this value to increase by 47% in 2050, which corresponds to 8.9 billion people (United Nations, 2009). The estimated population will aggravate the problems of climate change along with energy demands. Furthermore, energy agencies have shown that the global carbon dioxide emissions reached a staggering 35.7 billion tons in 2015 (Netherlands Environmental Assessment Agency, 2015). Similar reports have indicated that the current CO_2 levels exceed 390 ppm, and the CO_2 concentrations have been increasing by more than 3.30 ppm per year over the past decade (United Nations, 2015). Thus, if no effective measures are taken, the amounts of atmospheric CO_2 could reach 500 ppm in 2035 causing an alarming temperature increase of about 5 °C (International Energy Agency, 2015).

The effects of climate change are also being felt in South Africa i.e. there has been a drastic decline in the country's agricultural outputs due to low rainfall seasons and temperature rise (United Nations, 2009). Many parts of the country are experiencing drought and therefore are

no longer suitable for commercial farming. Climatologists have warned that climate change will have serious consequences on the following: (i) South Africa's coastal regions are expected to have an atmospheric temperature rise of 2 ^{o}C in 2050 and 4 ^{o}C by 2100, (ii) the country's interior regions are also expected to increase by 4 ^{o}C in 2050 and 7 ^{o}C in 2100, (iii) This will affect the country's food security, (iv) Alien invasive plants might increase and negatively affect the country's water resources, (v) This will likely exacerbate the health issues due to droughts and floods. Diseases such as malaria and cholera have been linked to extreme weather patterns, (vi) Bushlands and various commercial plantations will be vulnerable to wildfires (United Nations, 2009).

Therefore, diversification of energy fuels is an important requirement in the present global energy scenario (Nouni, 2012). Recent analysis of the world energy outlook suggests that renewable based technologies will provide a huge contribution to global energy provision within the next decades; currently they are only contributing about 15% of global energy supply (BP, 2013). Hence, this highlights a crucial need to promote their acceleration in order to boost the global energy supply and mitigate environmental pollution. Hydrogen is a promising energy option due to its properties which include high energy yield of 122 kJ/g and its carbon-neutral abilities (Cheng and Liu, 2011). These features make it an attractive fuel that can be used to reduce the heavy reliance on the fossil fuel economy (Elsharnouby et al., 2013; Wu et al., 2006). Presently, there are more than 400 projects globally that focus on the implementation of hydrogen-producing technologies. These initiatives form part of a global plan to boost energy security while mitigating environmental pollution by intensifying the hydrogen markets (Energy Information Administration, 2015). Hydrogen-producing technologies are also envisioned to increase significantly from 6% in 2020 to 50% in 2050. During this period, hydrogen infrastructures are expected to develop and become progressively more important in decarbonizing the current energy systems (Barry et al.,

2011). Hydrogen is commercially produced from thermochemical, photochemical, electrochemical, photocatalytic, and photoelectrochemical processes (Boboescu et al., 2016). The drawback of these processes is that they are expensive; contribute to greenhouse gas emissions, and uses high amounts of energy (Han and Shin, 2004). One attractive avenue for production of hydrogen is through biological methods. Biological hydrogen methods are advantageous because they are environmentally benign and cost-effective, thus being more competitive to thermochemical processes (Das and Veziroglu, 2001; Dong et al., 2009). The biological hydrogen routes include biophotolysis, photosynthetic and dark fermentation process. Dark fermentation is a preferred process because it can be conducted at moderate temperatures and pressure; it can use diverse feedstocks and microorganisms for its process. Moreover, dark fermentation process development has gained a tremendous impetus and governmental support in more than 30 countries worldwide (Meher Kotay and Das, 2008).

Therefore, this review provides an outlook on the energy sector in South Africa and highlights the need for implementation of clean and sustainable energy fuels. It comprehensively assesses the potential of using biowaste materials of agricultural, municipal and industrial process effluents for dark fermentative biohydrogen production in South Africa, while confronting their negative impacts on the environment. In addition, it critically evaluates the state-of-the-art and advancements in biohydrogen process infrastructure in South Africa. Finally, it discusses the technical challenges facing the dark fermentative biohydrogen economy and strategies that have been recommended for its scale-up.

2.2 Hydrogen energy

2.2.1 Its importance, application, and production methods

Reducing the reliance on hydrocarbon fuels and minimizing environmental pollution can only be realized by introducing clean and sustainable energy resources. Over the past few decades,

hydrogen has captured increasing global attention as an alternative to fossil fuels owing to its several merits which include (i) zero-carbon emissions, (ii) high energy yield, (iii) abundance, and (iv) diverse storage forms (e.g. gaseous, liquid, or coupled with metal hydrides). Most developed countries have therefore realized the future role of hydrogen and thus the concept of a "Hydrogen Driven Economy" was proposed by international hydrogen endorsement energy agencies such as the United States Department of Energy, European Hydrogen Association, and the International Partnership for Hydrogen Economy in efforts to intensify and commercialize its production (United States Department of Energy, 2015). The United States Department of Energy indicated in 2015 that it aims to invest about 35 million US dollars towards hydrogen infrastructure development projects as the country plans to reduce its dependence on foreign oil (United States Department of Energy, 2015). Hydrogen gas is extensively used in various industrial applications i.e. ammonia synthesis, methanol production, used in oil refineries for removal of impurities, used in processing of steel, electronic devices, and in desulfurization and reformation of gasoline. Furthermore, car manufacturers have now started to create vehicles that are powered by hydrogen fuel cells and are reported to be more effective than gasoline powered engines (United States Department of Energy, 2015). The global annual production of hydrogen is currently projected at 62 million tons, and has an annual growth rate of 8-10% (United States Department of Energy, 2015). Amongst the industrial hydrogen production processes, steam reforming of methane is an extensively used method. It produces nearly 50% of hydrogen; oil reforming produces nearly 30% of hydrogen, coal gasification yields about 18%, 3.9% comes from water electrolysis, and 0.1% from other methods (Saratale et al., 2008). However, these processes present a major challenge because they are energy intensive and contribute to greenhouse gas emissions as mentioned earlier. To alleviate the negative effects of fossil fuel utilization, hydrogen needs to be produced through clean and sustainable methods. In the past

few decades, researchers have started to look into biotechnological hydrogen production approaches such as biophotolysis, dark and photo-fermentation methods to yield cleaner hydrogen energy. The hydrogen- producing methods are summarized in Figure 2.1.

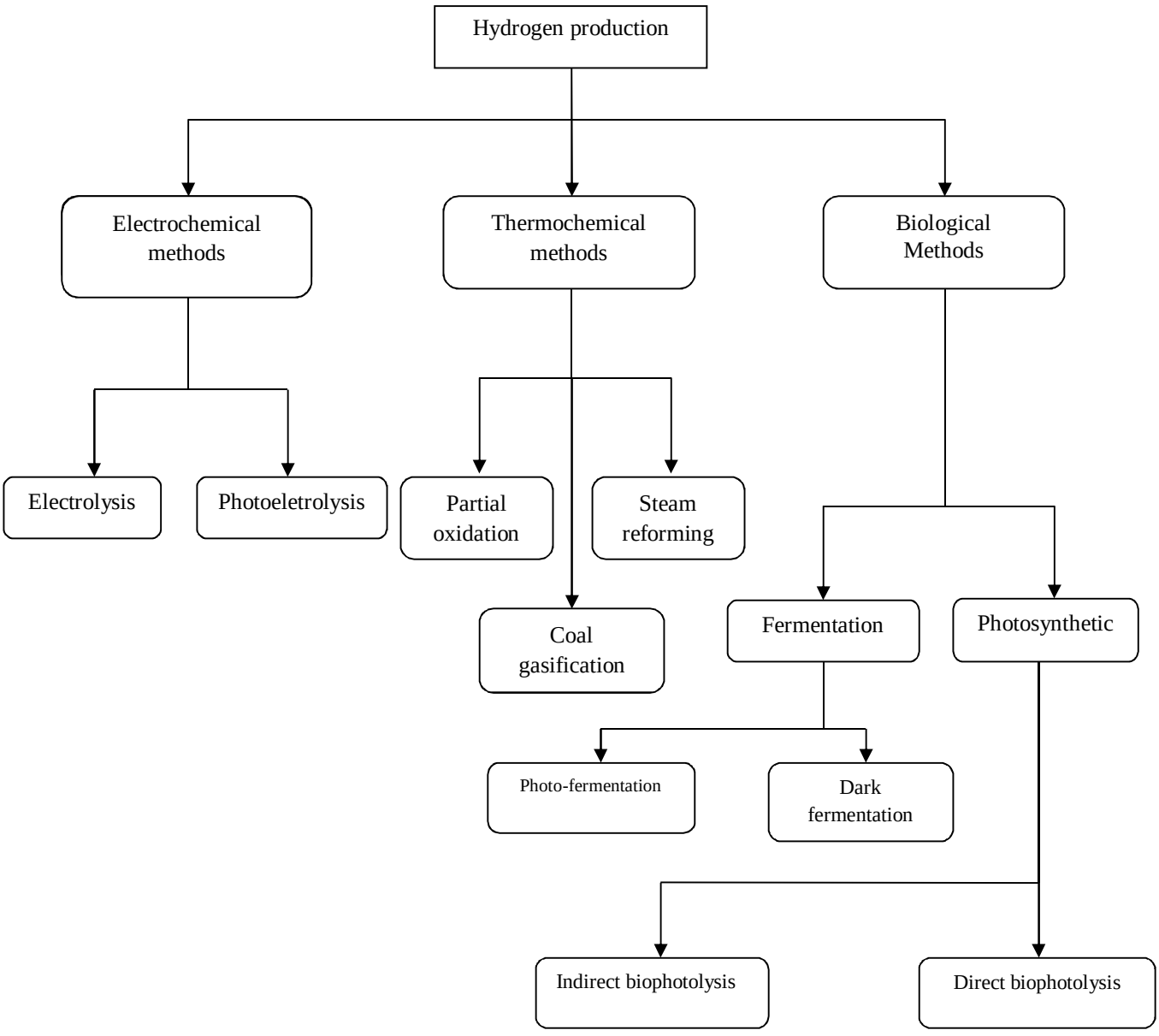


Figure 2.1: Hydrogen-producing methods (Saratale et al., 2008).

2.3 The energy sector in South Africa

2.3.1 Coal as a primary energy resource

South Africa is dependent on coal as its main energy source while the rest of the world is dependent on crude oil. Data from BP South Africa (Pty) Ltd showed that coal supplies approximately 72% of energy, followed by crude oil at 22% (BP, 2013). Other sources of energy such as nuclear, gas, and renewable fuels are only contributing less than 10% of total energy supply as illustrated in Figure 2.2 (BP, 2013). Moreso, coal is used by both the private and government sector for generation of electricity. There are five major companies that use more than 80% of the country's coal i.e. BHP Billiton, Anglo-American, Sasol, Exxaro, and Xstrata. The South African power parastatal Eskom is the largest producer of electricity in Africa and ranked amongst the top energy utilities in the world (Eskom, 2014), accounts for 70% of coal that is used for supplying the country's electricity (Figure 2.3). The extensive use of coal as a primary energy fuel is due to its widespread availability. South Africa has 19 coal mines that are situated in the provinces of the Eastern Cape, North West, Limpopo, KwaZulu-Natal, Free State, Mpumalanga, and Gauteng (Jeffrey, 2005). However, some of these mines have been abandoned due to depletion of coal reserves (see Figure 2.4) (Jeffrey, 2005).

2.3.2 Shortcomings of coal energy

Several reports have highlighted that South Africa's dependence on coal will cause these reserves to be exhausted sooner than anticipated. For example, de Jager (1978) postulated these reserves at 58.4 billion tons. Thereafter, Bredell (1987) forecasted them at 55.3 billion tons. The Department of Mineral Resources projected them at 33.8 billion tons in 2000. A further decline was confirmed by Hartnady (2010); they were predicted at 15 billion tons. South Africa produces significant amount of carbon dioxide i.e. it generated about 1.4% of

CO_2 globally and 40% of CO_2 within the continent in 2011, therefore making it the highest in Africa and 14th in the world (International Energy Agency, 2013). Moreso, the country's energy consumption has drastically increased the levels of CO_2 emissions by 18% from 2001 to 2011 (Hartnady, 2010). South Africa's power parastatal (Eskom) has been facing an immense pressure as a result of the country's escalating energy demands. The power utility is presently functioning at near full-scale i.e. it has a production capacity of 40 gigawatts whereas the country's peak demand is 36 gigawatts (International Energy Agency, 2013). This has caused persistent power shortages and blackouts which resulted in an economic decline of approximately 282 million US dollars (Energy Information Agency, 2013). This crisis is exacerbated by fact that the country's coal stations are old and thus regularly need maintenance and also have a small capacity (Sekoai and Daramola, 2015).

2.3.3 South Africa's alternative energy policy

South Africa has massive clean alternative energy resources like biomass, wind, solar, and marine energy that could be used in the mitigation of carbon dioxide emissions, and improve the country's energy security (Eberhard, 2011). Therefore, the Department of Energy emphasized the need to diversify the country's energy mix in order to curb the problems associated with energy derived from coal. This triggered the formation of South African National Energy Development Institute (SANEDI), which is an organization formed in 2008. The main purpose of SANEDI is to implement and propose policies and frameworks for strategies of alternative and sustainable energy development in South Africa by collaborating with various stakeholders such as private, government and academic institutions. In addition, its role is to ensure that South Africa has the necessary skills, expertise, and resources for implementation of alternative energy based technologies to address the country's economic, environmental, and social needs (Eberhard, 2011).

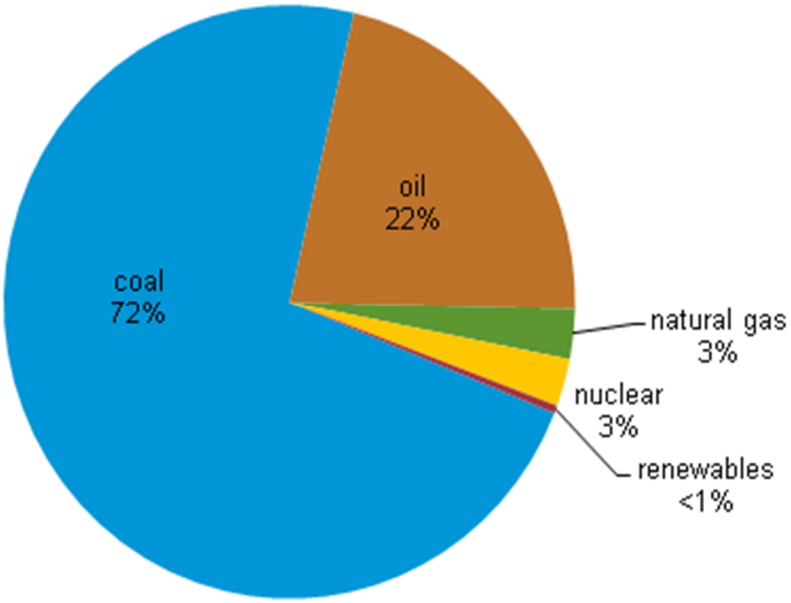


Figure 2.2: Energy utilization in South Africa (BP, 2013).

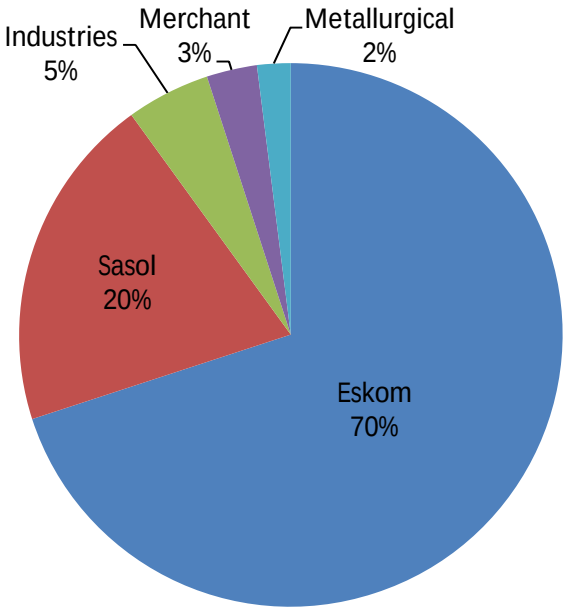


Figure 2.3: Coal usage in South Africa (Eberhard, 2011).

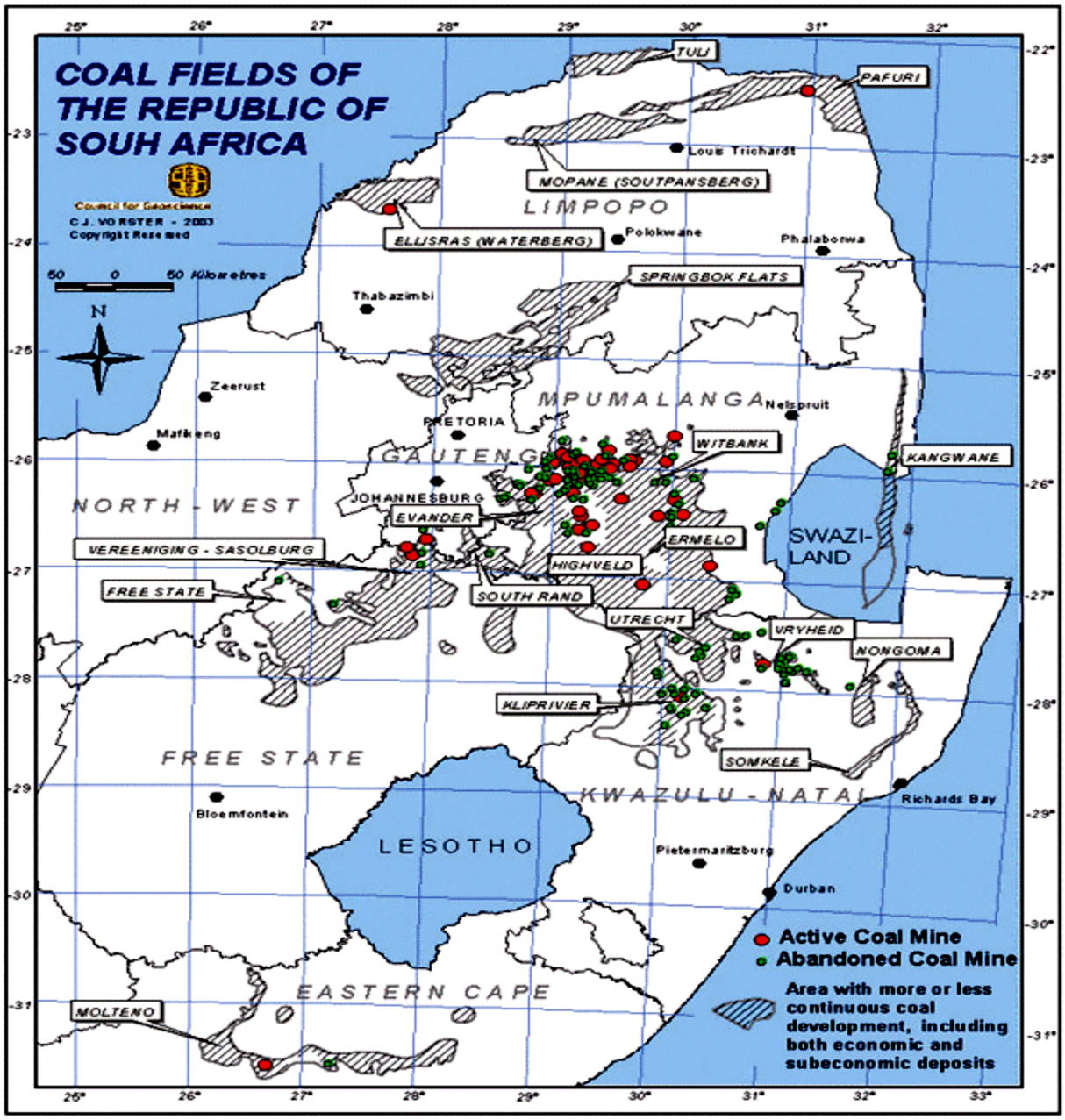


Figure 2.4: Map showing the distribution of coal mines in South Africa. Active mines are indicated with red dots and abandoned mines are indicated with green dots (Fourie et al., 2006).

2.4 Types of alternative energy used in South Africa

South Africa is currently using various forms of renewable energy resources which include nuclear, wind, and solar energy. These technologies are elaborated below.

2.4.1 Solar energy

Solar resources include solar water heaters for hot water supply and solar power for generating electricity. The potential for solar water heaters is huge in South Africa, studies show that approximately 400 000 homes are installed with solar water heaters every year (Fluri, 2009). It has been shown that about 4% of residential electricity consumption results from heating of geysers. Moreover, their application is motivated by the socio-economic needs for energy security, environmental sustainability, and reducing the usage of electricity. This technology is currently being applied in other countries such as China (Rizhao) where 99% of households are reported to be using solar water heaters (Winkler, 2010). The Department of Energy in South Africa proposed a 5 million long-term plan of installing solar water heaters across the country by 2020. With regards to the utilization of solar power for electricity generation, Eskom installed a 25 kW solar panel as part of the initiatives from the South African government to assess this technology. Besides, Eskom joint collaboration with the University of Stellenbosch resulted in the construction of the SKA Meerkat Radio Telescope Array (Northern Cape, South Africa) which began in 2012 (Winkler, 2010).

2.4.2 Wind energy

In recent years, development of wind projects has been increasing in South Africa. In 2014, the country launched one of its biggest wind farms in Africa. The Jeffrey's Bay Wind Farm located near Humansdorp in the province of Eastern Cape was built by the British based company Globleleq (Pty) Ltd. The farm comprises of 60 (80 metre high) wind turbines which are spread over 3700 hectares and can produce up to 138 megawatts of electricity (Banks and Schaffler, 2006). Other projects include the Klipheuwel Wind Energy Demonstration Facility (KWEDF) which has a total capacity of 3.2 megawatts (Winkler, 2010).

2.4.3 Nuclear energy

The South African government is in the process of building new nuclear power plants in the country. Two nuclear reactors which are currently operating in Koeberg account for 4% of the country's electricity supply. However, the country intends to generate 9600 megawatts from the new nuclear power plants that are about to be constructed (Brodski, 2009).

2.5 Integration of biofuels into South Africa's energy mix

South Africa aims to strengthen its alternative energy options in order to cope with high energy demands and reduce its carbon footprint. Diversification of alternative energy resources will assist the country to reduce the high costs of imported petroleum oil. Thus, biofuel production technologies have the potential to expand and diversify South Africa's energy supply, which will in turn reduce the country's dependence on dwindling coal reserves and intensify its energy supply. Furthermore, biofuel development initiatives are gaining increasing momentum in developing countries like South Africa and are foreseen as a catalyst for (i) infrastructural development projects, (ii) reducing high international oil prices, (iii) boosting the country's energy sector, (iv) and creation of employment opportunities (Sekoai and Yoro, 2016).

2.6 Biofuel development initiatives in South Africa

Biofuels contributes up to 14% of energy in South Africa (Blanchard et al., 2011). Biomass derived energy is extensively used by rural and other low-income urban households to generate fuel that is used for cooking and heating. It is also used in boilers by various South African industries to generate electricity (Blanchard et al., 2011). The Department of Energy announced in 2013 that it aims to begin a regulatory blending process of diesel and petrol with biofuels as from 2015; this is intended to stabilize the country's biofuel sector thereby reducing its reliance on hydrocarbon fuel (Blanchard et al., 2011). In addition, it also

proposed a five-year pilot-phase plan which is aimed at achieving 2-5% of biofuels. To date, five companies have been granted licenses to produce bioethanol and biodiesel in South Africa. Analysis of potential feedstocks that can be used revealed that sorghum is suitable for bioethanol production while soybeans are potential feedstocks for biodiesel production (Blanchard et al., 2011). However, maize has been excluded from these feedstocks because it is one of the country's staple foods and this may affects the country's food security. Other biofuel development initiatives include the Bronkhorstspruit Biogas Plant which is owned by the Bio2Watt Company. It is the leading commercial-scale biogas producer in South Africa and uses approximately 120 000 tons of biowaste effluents to generate biogas (Bio2Watt, 2016). It has partnered with a leading car manufacturer (BWW, South Africa) which uses the biogas in their production plant. Moreover, as South Africa is experiencing a huge influx of biomass generated from the agricultural, municipal, and industrial sector; other potential biofuel options such as dark fermentative biohydrogen production will contribute enormously to the intensification of cleaner energy production in the country.

2.7 Biohydrogen production potential in South Africa

2.7.1 The potential of dark fermentative biohydrogen production in South Africa

Recently, South Africa has been focusing on the implementation of other biofuel options such as dark fermentation process because of its non-polluting and waste beneficiation characteristics (Sekoai and Daramola, 2015). Dark fermentation from biowaste effluents is advantageous in South Africa because the country is experiencing an enormous burden with regards to its waste management methods i.e. thus the concept of "waste-to-energy" has been gaining increasing support from various stakeholders within the country. Secondly, dark fermentation uses diverse biowaste effluents (e.g. agricultural, industrial, and municipal) which are abundantly available and are causing a disposal challenge. The utilization of these

effluents makes this process economically viable in contrast to other energy generating methods. Other biohydrogen production methods include photo-fermentation, direct, and indirect biophotolysis (Chandrasekhar et al., 2015; Ghimire et al., 2015; Kumar and Chowdhary, 2016). However, dark fermentation is a highly favoured process because of its simplicity, cost-effectiveness, and sustainability. In addition, this biotechnological process has attracted increasing attention among researchers in South Africa (Obazu et al., 2015; Faloye et al., 2014; Hassan and Gueguim Kana, 2016; Mafuleka and Gueguim Kana, 2015; Moodley and Gueguim Kana, 2015; Ngoma et al., 2011; Sekoai and Gueguim Kana, 2014a, b; Sekoai et al., 2016; Sewsynker and Gueguim Kana, 2015).

2.7.2 State-of-the-art and biohydrogen process advancement in South Africa

Hydrogen based infrastructures are under serious consideration in South Africa in efforts to develop cleaner, reliable and sustainable energy fuels. A ten-year innovation plan was proposed by the Department of Science and Technology in 2008. This strategic plan involved the development of alternative energy resources that would assist in reducing carbon emissions and also meet the country's high energy demands. Therefore, Hydrogen South Africa (HySA) was established in the same year (Hydrogen South Africa, 2016). The purpose of HySA is to develop innovation towards the implementation of hydrogen technologies in South Africa. It consists of three centres of competence which are HySA Infrastructure, HySA Catalysts, and HySA Systems. These research centres are co-hosted by five institutions namely Mintek, University of Cape Town, North West University, University of Western Cape, and Council for Scientific and Industrial Research (CSIR) (Hydrogen South Africa, 2016). The HySA Infrastructure focuses on the development of hydrogen production technologies through small and medium-scale hydrogen producing reactor prototypes (Hydrogen South Africa, 2016). The group is also researching on hydrogen storage materials. HySA Catalysts is a joint research collaboration between Mintek and the University of Cape

Town; they are responsible for developing industrial value chain catalysts that will enhance hydrogen fuel cell technologies. The HySA Systems aims to develop and improve hydrogen-based technologies and is chaired by the University of the Western Cape. Its objectives are to (i) develop hydrogen fuelled vehicles system prototypes, and (ii) conduct validation and hybrid processes within the HySA research centres which are (i) combined heat and power, (ii) miniaturized bioprocess systems, and (iii) hydrogen fuelled cars (South African Institute for Advanced Materials Chemistry, 2016). Therefore, establishment of HySA could pave a way for the advancement of hydrogen markets in South Africa.

Despite the biohydrogen development initiatives that have been carried out by various research institutions, this technology is still in the Research and Development stages in most countries including South Africa, implying that most biohydrogen production studies have been carried out at bench-scale by various researchers across South Africa (Obazu et al., 2015; Faloye et al., 2014; Hassan and Gueguim Kana, 2016; Mafuleka and Gueguim Kana, 2015; Moodley and Gueguim Kana, 2015; Ngoma et al., 2011; Sekoai and Gueguim Kana, 2014a, b; Sekoai et al., 2016; Sewsynker and Gueguim Kana, 2015). This prompts the need for extensive large-scale processes in order to fully understand the process dynamics (e.g. setpoint conditions, partial pressure, heat transfer, mass transfer, etc) involved during its production and this will provide reliable scalable data that could be used towards its industrialization.

2.8 Biowaste production in South Africa

Over the past years, South Africa witnessed a drastic increase in waste production due to the high level of urbanization and industrialization that is occurring in most cities across the country. The total waste distribution data for South Africa in 2014 is shown in Table 2.1; an estimated 7.80 million tons of waste was produced by the municipal sector. The agricultural

sector generated 2.95 million tons, whereas the industrial sector generated 12.1 million tons of waste (Department of Environmental Affairs, 2014). The amount of biowaste generated by each province is also presented in Table 2.2. It is apparent from this data that South Africa is experiencing a significant growth in waste volumes. As a result, 42.3 million tons of organic municipal waste was generated in 1997 and this value increased to 69 million in 2014. During this period, the production of biowaste rose by 63.1%. Data from the Department of Environmental Affairs have also indicated that waste volume in South Africa increases by approximately 11 million tons each year (Department of Environmental Affairs, 2014). Therefore, biowaste materials will present an enormous burden on the environment and people if it is not properly managed. Waste beneficiation approaches such as dark fermentative biohydrogen production will significantly assist to curb environmental pollution while generating clean and sustainable energy.

Table 2.1: Total waste distribution data in South Africa (tons) (Department of Environmental Affairs, 2014).

Waste type	Produced	Recycled	Disposed	% Recycled
Municipal	7 800 328	-	7 800 328	0
Agricultural	2 954 461	1 034 061	1 920 400	35
Industrial	12 120 783	9 255 376	2 865 407	76
Saltwater	4 166 129	-	4 166 129	-
Fly ash and dust	31 420 488	1 885 229	29 535 259	6
Bottom ash	5 385 968	-	5 385 968	-
Slag	5 000 150	2 500 075	2 500 075	50
Mineral	335 000	-	335 000	-
Electronic	66 321	6 975	59 346	11
Sewage sludge	657 963	125 013	493 472	19
Miscellaneous	327 250	-	327 250	-
Construction	4 735 142	766 017	3 969 125	16
Paper	1 649 257	938 900	710 357	57
Plastic	1 287 701	240 162	1 047 539	19
Glass	938 769	301 218	637 551	32
Metals	3 181 213	2 466 960	714 253	78
Tyres	245 633	9 866	235 767	4
Other	36 161 137	-	36 161 137	0

-: not available

Table 2.2: Biowaste generated by the nine provinces (Department of Environmental Affairs, 2014).

	1997		2014		1997-2014	1997-2014
Province	**Tons**	**%**	**Tons**	**%**	**Total growth %**	**Annual average growth %**
Eastern Cape	2 382 000	5.6	3 215 929	4.6	35.0	2.9
Free State	1 667 000	9.9	3 887 381	5.6	133.2	7.3
Gauteng	17 899 000	42.3	26 085 304	37.8	45.7	3.2
KwaZulu-Natal	4 147 000	9.8	5 754 823	8.3	38.7	3.2
Limpopo	3 781 000	8.9	11 320 317	16.4	199.4	9.6
Mpumalanga	738 200	1.7	986 392	1.4	33.6	2.4
Northern Cape	1 487 321	3.5	2 482 874	3.6	66.9	4.4
North West	1 652 100	3.9	2 289 499	3.3	38.6	3.0
Western Cape	8 553 000	20.2	12 989 885	18.8	51.9	3.6
Total	**42 306 621**	**100**	**69 012 404**	**100**	**63.1**	**4.3**

2.9 Elemental composition of South African biowaste effluents

As a preliminary investigation, we conducted analysis of carbon, hydrogen, nitrogen, sulphur, and oxygen elements contained in South African biowaste effluents in our laboratory using a Flash 2000 CHNS/O Analyzer (Thermo Scientific, USA). Oxygen (wt. %) was calculated by the difference of C, H, N, S, which was subtracted from 100. The chosen effluents are highly abundant in South Africa and form a substantial fraction of the country's biowaste materials. Thus, organic composition of these effluents is crucial because they affect the overall microbial conversion yields of biohydrogen production. Furthermore, these elements also affect the activity of biohydrogen-producing hydrogenase enzymes (Kapdan and Kargi, 2006). The C, H, N, S, and O composition is shown in Table 2.3.

Table 2.3: CHNSO composition of South African biowaste effluents.

Elemental composition (%)	C	H	N	S	O
Apple	42.58	6.51	0.36	-	50.55
Bread	40.47	5.94	1.81	-	51.78
Brewery	42	6.19	2.01	-	49.8
Cabbage	39.46	5.45	3.42	1.05	50.62
Corn-cob	42.8	5.88	0.49	-	50.83
Kitchen	43.8	6.53	2.36	-	47.31
Mango	53.74	8.05	1.03	-	37.18
Pear	41.89	6.45	0.33	-	51.33
Potato	40.45	5.86	0.3	-	53.39
Sugarcane	42.22	6.37	0.3	-	51.11

-: not detected.

2.10 Disposal challenges associated with biowaste effluents in South Africa

In South Africa, major cities are experiencing increasing population growth due to high level of urbanization and industrialization as highlighted earlier. Moreso, there is a rapid infrastructure development occurring in these cities in order to cater for the needs of its inhabitants. As a result, there has been a sporadic increase in the generation of biowaste

materials. Biowaste materials of agricultural, municipal, and industrial effluent pose serious health risks on people living in these sites. Landfill sites have been underlined as the possible cause of birth defects and respiratory illness such as asthma (Broomfield et al., 2004). Incinerators have also been linked to these illnesses. Moreover, composting and material recycling facilities have been linked to odours and lung related diseases such as bronchitis (Broomfield et al., 2004). The Department of Health also raised concerns about the disposal of these effluents because they attract disease vectors such as mosquitoes, flies, and rats to breed in landfills (Department of Health, 2014).

From an environmental standpoint, biochemical decomposition reactions produce substantial amounts of greenhouse gases (e.g. methane and carbon dioxide) on landfills and are released into the atmosphere. It has been reported that other toxic gases such as ammonia are formed during biodegradation of biowaste materials (Eklund et al., 1998; Kemfert and Schill, 2009). A study by Viitez et al. (2000) indicated that the biological conversion of biowaste on landfills occurs at a slow rate and it could take years to complete i.e. the authors reported that anaerobic digestion reactions on landfills may extend up to 20-40 years and this poses serious detrimental effects on the environment (Viitez et al., 2000). It has also been shown that the disposal of these effluents will increase in developing nations like South Africa faster than in less developed regions, due to rapid infrastructure development that is occurring in these regions (Broomfield et al., 2004). In other related studies, Devesa-Rey et al. (2009) showed that the costs of recycling these effluents and the penalties imposed on companies have increased significantly in recent years, often reaching millions of dollars. These fines are sometimes combined with other penalties, such as the obligation to decontaminate polluted areas which can involve considerable expenses for companies. In this regard, the South African Environmental Legislation mandates government municipalities and industries to dispose their effluents in a manner that will not cause a threat to people and the environment.

Nonetheless, the current waste disposal methods do not comply with these regulations, implying that new and innovative approaches for biowaste management are needed to address these challenges.

2.11 Feasibility of biowaste effluents for dark fermentation in South Africa

Studies in literature have assessed the potential of various carbon sources such as glucose (Van Ginkel et al., 2001), sucrose (Chen et al., 2001; Khanal et al., 2004), and xylose (Lin et al., 2006; Lo et al., 2009) on dark fermentative biohydrogen yields. Even though this process is well researched from these sugars, utilization of these substrates is too expensive to support the dark fermentative "biohydrogen driven" economy i.e. the cost of substrates account for approximately 60% of the overall bioprocess costs (Argun and Dao, 2016). Therefore, the use of biowaste effluents for its production will significantly enhance its process economics because these feedstocks are readily available, considered waste materials, and possess high hydrogen efficiency. Feedstocks such as food materials are highly favoured substrates because they are rich in nutritional composition i.e. 80-95% volatile solids, and 75-85% moisture, thus favouring the enumeration of dark biohydrogen-producing bacteria during dark fermentation (Guo et al., 2010; Kim et al., 2004; Shin et al., 2003; Zhou et al., 2013).

The latent energy present in these effluents can be recovered via microbial bioprocesses to produce biohydrogen. The potential of using these effluents for dark fermentation is highly documented in literature (Dong et al., 2009; Elbeshbishy et al., 2011; Lay et al., 1999). Examples of dark fermentation yields reported are 138 ml H_2/g VS, 92 ml H_2/g TVS, 126.9 ml H_2/g TVS, 183 ml H_2/g TVS, 189 ml H_2/g COD, and 78 ml H_2/g COD, respectively. These studies were conducted at different operational conditions of temperature (30-48 ^{o}C) and pH (5-6), deemed favourable for biohydrogen fermentation studies (Shin et al., 2003; Zhou et al., 2013). In addition, other associated substrates such as wastewaters from food

processing industries have a great potential for dark fermentation due to their nutritional content. For example, South Africa is listed amongst the top seven wine producers in the world, and therefore the wine industry yields large quantities of wastewater each year. Approximately one billion litres of wastewater is produced from more than three thousand wine producers in South Africa (Sheridan et al., 2010). Wastewater from wine industries is rich in COD (300 - 60 000 mg/l), has a pH range of 3 - 8, and consists of various trace elements (Ca, K, Na, and Mg) which makes it an ideal substrate for dark fermentation process (Buys, 2015). Other huge sectors such as the sugarcane industry (generates up to 20.6 million tons of sugarcane per annum) produce large volumes of molasses which has a high concentration of fermentable sugars and COD (50–100 g/l) (Jimenez et al., 2004). Several researchers assessed the biohydrogen production potential from wastewaters; Lin et al. (2011) studied the effect of food processing wastewaters of fructose and molasses on dark fermentation, and obtained a biohydrogen yield of 167 ml H_2/g COD for wastewater of fructose and 187 ml H_2/g COD for wastewater of molasses respectively. Van Ginkel et al. (2005) investigated dark fermentative biohydrogen production from different wastewaters (potato, apple pomace, and confectioners), and reported a high yield of 210 ml H_2/g COD from potato wastewater. These studies present a viable approach towards an economically feasible dark fermentative biohydrogen production based on the beneficiation of waste. Table 2.4 shows various studies that have utilized agricultural, municipal, and industrial biowaste materials for dark fermentative biohydrogen production. The biohydrogen production yields varied due to several factors such as (i) inoculum type, (ii) operating conditions, (iii) bioreactor design, (iv) type of substrate, and (v) working volume. Hence, this review presents strategies for optimizations of dark hydrogen fermentations from these biowaste effluents which are discussed in section 2.17.2.

Table 2.4: Biohydrogen fermentation processes from various biowaste materials.

Inoculum	Substrate	H_2 yield	% H_2	Reference
Anaerobic sludge	Food waste	38 ml H_2/g VS	49.1	Angeriz-Campoy et al. (2015)
Seed sludge	Food waste	70.7 ml H_2/g TVS	-	Algapani et al. (2016)
Bacillus sp.	Organic waste	61 ml H_2/g VS	-	Shah et al. (2015)
Aspergillus Awamori	Bread waste	7.4 H_2 L/Ld	-	Wei et al. (2016)
Anaerobic sludge	Potato waste	171.1 ml H_2/g VS	-	Ghimire et al. (2015)
Anaerobic sludge	Paper waste	140 ml H_2/g total sugar	-	Eker and Sarp (2016)
Anaerobic sludge	Potatoes	106 ml H_2/g VS	41-55	Dong et al. (2009)
Anaerobic sludge	Lettuce	50 ml H_2/g VS	37-67	Dong et al. (2009)
Mixed cultures	Rice waste	2.14 mol H_2/mol hexose	53-61	Yu et al. (2002)
Sewage sludge	Biosolids	10 ml H_2/g COD	-	Wang et al. (2003)
Clostridium + *Enterobacter*	Sweet potato (5%)	7.0 mol H_2/mol glucose	-	Yokoi et al. (2002)
Clostridium + *Enterobacter*	Sweet potato (2%)	4.5 mol H_2/mol glucose	-	Yokoi et al. (2002)
Mixed cultures	Fructose wastewater	166.8 ml H_2/g COD	-	Lin et al. (2011)
Mixed cultures	Molasses wastewater	187 ml H_2/g COD	-	Lin et al. (2011)
Sewage sludge	Food waste	205 ml H_2/g VS	52-56	Chu et al. (2008)
Sewage sludge	Mixed organic waste	52.5-71.3 H_2 L/kg VS	-	Gomez et al. (2006)
Anaerobic sludge	Food waste	97 ml H_2/g VS	-	Elbeshbishy et al. (2011)
Anaerobic digester	Food waste	1-2.3 mol H_2/mol hexose	43.9-51.4	Lee et al. (2010)
Anaerobic digester	Food waste	96-114 ml H_2/g VS	-	Cappai et al. (2009)
Anaerobic sludge	Mixed organic waste	76 ml H_2/g COD	-	Zhou et al. (2013)

Table 2.4 continued.

Caldicellulosiruptor	Maize leaves	18 ml H_2/g TVS	-	Ivanova et al. (2009)
Caldicellulosiruptor	Sweet sorghum	32.4 ml H_2/g VS	-	Ivanova et al. (2009)
Caldicellulosiruptor	Bagasse	19.6 ml H_2/g VS	-	Ivanova et al. (2009)
Anaerobic sludge	Cabbage	26.3-61.7 ml H_2/g TVS	-	Okamoto et al. (2000)
Anaerobic sludge	Carrot	44.9-70.7 ml H_2/g TVS	-	Okamoto et al. (2000)
Anaerobic sludge	Rice	19.3-96.0 ml H_2/g TVS	-	Okamoto et al. (2000)
Digested sludge	Potato	30 L H_2/kg TS	45	Zhu et al. (2008)
Mixed cultures	Wheat starch	92 mol H_2/g starch	-	Zhang et al. (2003)
Anaerobic sludge	Sugarbeet juice	1.9 mol H_2/mol hexose	-	Hussy et al. (2005)
Mixed cultures	Sweet sorghum	10.4 L H_2/kg TS	-	Antonopoulou et al. (2008)
Heat treated soil	Apple processing waste	0.9 L H_2/g COD	-	Van Ginkel et al. (2005)
Heat treated soil	Potato processing waste	2.8 L H_2/g COD	-	Van Ginkel et al. (2005)
Digested sludge	Cheese whey	0.83 mol H_2/mol glucose	-	Venetsaneas et al. (2009)
Anaerobic sludge	Cassava waste	851.84 ml H_2/h	-	Sangyoka et al. (2007)
Activated sludge	Cornstalk	126.22 ml H_2/g CS	-	Wang et al. (2010b)
Dairy manure	Corncob	757.7 ml H_2/g COD	-	Yang et al. (2010)
Clostridium butyricum	Sugarcane bagasse	1.73 mol H_2/mol TS	-	Pattra et al. (2008)
Anaerobic sludge	Corn starch	0.51 mol H_2/mol glucose	-	Arooj et al. (2008)

-: not available

2.12 Categorization of biohydrogen-producing biowaste materials

2.12.1 Agricultural waste

Agricultural residues consist mainly of lignocellulose materials which are abundantly available in South Africa. They are economically feasible because they are inexpensive and easily accessible feedstocks (Mafuleka and Gueguim Kana, 2015). However, these waste materials create a disposal challenge in most countries including South Africa because most of them have a slow degradation process and contain high mineral content. Hence, they are mostly burnt which increases air pollution and jeopardizes human health. The plant biomass of these substrates consists of lignin, cellulose, and hemicellulose which must undergo vigorous pretreatments to release the fermentable sugars (e.g. glucose, galactose, etc). Examples include bean husks, grasses, corn cobs, wheat straw, and other materials (Wu et al., 2006).

2.12.2 Organic Fraction of Municipal Solid Waste

Food waste consists of a large proportion of Organic Fraction of Municipal Solid Waste (OFMSW); it is rich in nutritional content (85-95% volatile solids and 75-85% moisture) and its nutritional characteristics make it an ideal substrate for biohydrogen production (Han and Shin, 2004). It also comprises of other fermentable rich materials that are found in raw and cooked food products that are discarded in recycle bins and landfills. However, it poses an environmental challenge because it generates odours and pests (Han and Shin, 2004).

2.12.3 Industrial waste

Industrial waste includes effluents from sugar refineries, cereals, cheese, brewery, paper, and beverage processing companies. These industries produce large quantities of wastewater which contains sugars and starches. Thus, this favours the production of biohydrogen which is generated by a series of biochemical pathways manifested by acidogenic bacteria such as

Clostridium and *Bacillus* species (Han and Shin, 2004). The exploitation of wastewaters for dark fermentative biohydrogen production provides a platform for generation of clean energy while removing contaminants in water (Dong et al., 2009). Moreover, utilization of wastewaters for energy production is beneficial because it does not generate environmental pollution, and there is simultaneous energy recovery (Dong et al., 2009).

2.12.4 Other types of biowaste substrates

Besides the abovementioned substrates, other feedstocks that have been used in dark fermentative biohydrogen production include:

- Livestock manure (Cavinato et al., 2015; Yin et al., 2014).
- Perennial grasses (Mafuleka and Gueguim Kana, 2015; Lewis et al., 2015).
- Algal biomass (Park et al., 2009; Roy et al., 2014).
- Waste sludge (Lin et al., 2011; Wang et al., 2003).

All of these biowaste feedstocks are classified in Figure 2.5. Municipal and industrial effluents are ideal substrates for biohydrogen-producing bacteria because they contain low lignin content and they are also rich in carbohydrate composition as compared to agricultural waste, which requires various pretreatment methods in order to access the fermentable sugars (Dong et al., 2009).

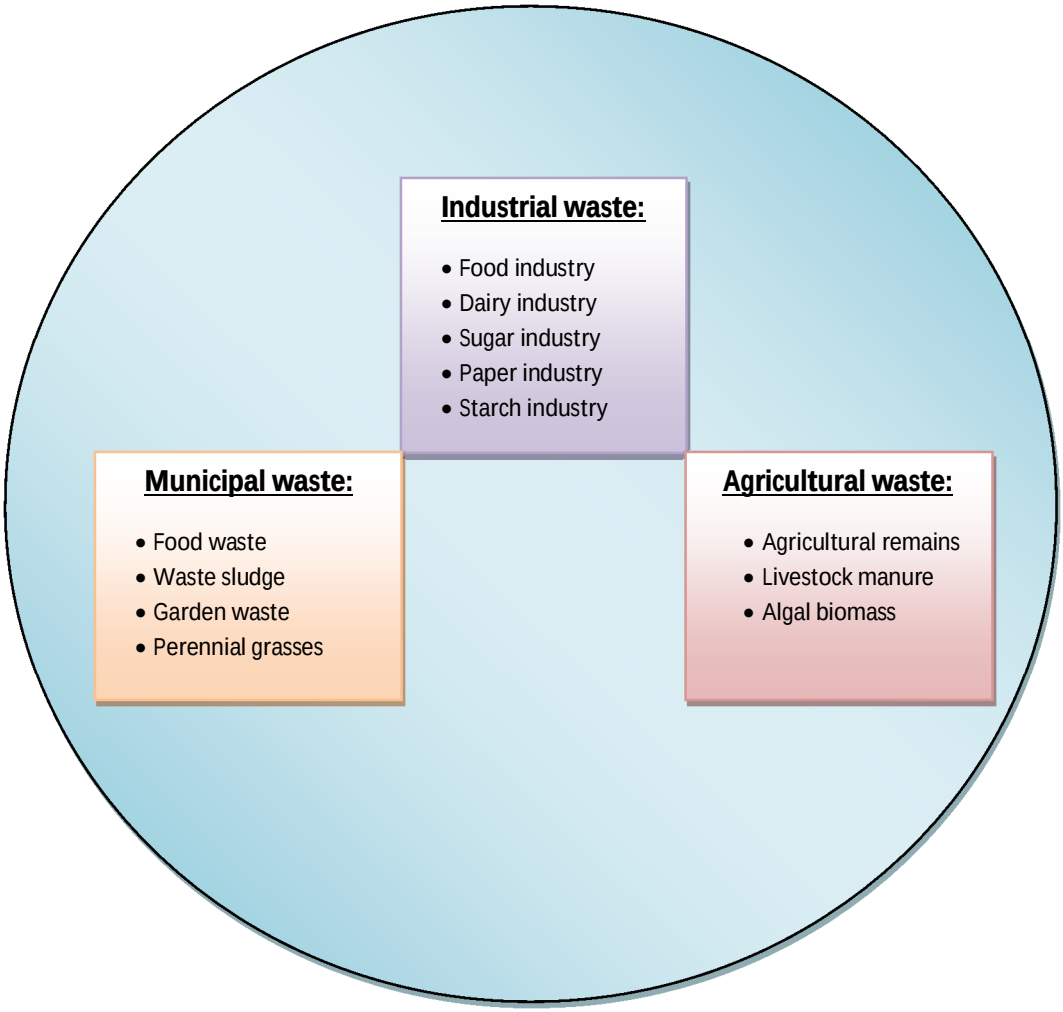


Figure 2.5: Classification of biohydrogen-producing biowaste substrates.

2.13 Key parameters affecting dark fermentation from biowaste effluents

Dark fermentative biohydrogen production processes are governed by various operating parameters. These parameters need to be operated at optimum conditions for enhanced biohydrogen yields.

2.13.1 Temperature

Temperature is considered as the most important process parameters in biohydrogen fermentation. It affects the growth rate and metabolic pathways in fermentative biohydrogen-producing bacteria (Elsharnouby et al., 2013), which in turn influences the activity of

biohydrogen-producing enzymes such as hydrogenases and nitrogenases (Khanna and Das, 2013). In addition, some studies have shown that temperature plays a crucial role in substrate utilization, volatile fatty acids accumulation, hydrogen conversion efficiency, and microbial consortia during dark fermentation processes (Fang et al., 2002; Lay et al., 1999).

Biohydrogen fermentation can be conducted at mesophilic (20-42 ^{o}C), thermophilic (42-75 ^{o}C) or hyperthermophilic conditions (>80 ^{o}C) (Sinha and Pandey, 2011). However, mesophilic temperatures are highly recommended in biohydrogen processes since they are cost effective and require relatively low levels of energy. A review by Elsharnouby et al. (2013) indicated that approximately 60% of biohydrogen production studies are carried out under mesophilic conditions. However, one of the drawbacks of biohydrogen production at mesophilic conditions is low hydrogen conversion efficiency. Classen et al. (2000) revealed that the conversion of acetate to biohydrogen is thermodynamically unfavourable at mesophilic temperatures. Therefore, thermophiles have a great potential for increased hydrogen conversion efficiency since the process is thermodynamically favourable. Moreover, thermophiles can inhibit biohydrogen-consuming methanogens during dark fermentation processes (De Gioannis et al., 2013). Nonetheless, this process is not commercially feasible due to high energy demands and could hinder the development of a large-scale biohydrogen production.

2.13.2 pH of the fermentation medium

pH is also considered as one of the most crucial process parameters in biohydrogen fermentation studies. It affects hydrogenase activity, metabolic activity, and substrate hydrolysis (De Gioannis et al., 2013). Protons (H^{+}) are essential for maintaining optimum levels of adenosine triphosphate (ATP) within biohydrogen-producing bacteria. Thus, an ideal pH is significant since it is responsible for the uptake of nutrients, proton gradient, and

polarity during biohydrogen fermentation processes. Hence, many studies have shown that pH should be operated at optimum conditions to prevent the growth of biohydrogen-consuming methanogens (Pan et al., 2008).

Different pH values ranging from 4-9 have been reported in biohydrogen production studies, as a result of several contributing factors such as type of substrate, microbial consortia, and process conditions (Elsharnouby et al., 2013). Most biohydrogen fermentation experiments are carried out without pH control. Previous studies have shown that the optimum pH range for optimal biohydrogen yield or specific biohydrogen production rate is 5.2-6.0 using either pure or mixed cultures (Oh et al., 2004; Zhang et al., 2008). Several biohydrogen producing processes reported that the initial pH values of 5.5-7.5 may represent the optimum and acceptable range for biohydrogen fermentations (Argun et al., 2008; Hawkes et al., 2002; Kim et al., 2011; Wu et al., 2006; Yasin et al., 2011). However, some studies have revealed that low pH values (below 4.5) inhibit the hydrogenase activity during dark fermentation process (Fang et al., 2002; Hawkes et al., 2002; Khanal et al., 2004).

It has been established that biohydrogen production occurs during the acidogenic stage via acetic and butyrate fermentation pathways (Van Ginkel et al., 2005). During this process, biohydrogen producing clostridia grow exponentially at pH 5.5-6.5 (Van Ginkel et al., 2005). Thereafter, there is a decline in biohydrogen production due to microbial switch from acidogenesis to solventogenesis caused by accumulation of fermentative by-products such as volatile fatty acids (VFAs), methanogens and alcohols. These end-products change the buffering capacity of the medium and are observed at pH below 4.5 (Khanal et al., 2004; Venkata Mohan et al., 2008).

2.13.3 Hydraulic Retention Time

Hydraulic Retention Time (HRT) is regarded as an important control parameter affecting continuous biohydrogen production processes (Zhang et al., 2006). HRT needs to be regulated during biohydrogen fermentation processes in order to inhibit biohydrogen-consuming bacteria (Lin and Lay, 2004). The choice of HRT in biohydrogen production is dependent upon the type of substrate used. However, short HRTs are ideal for biohydrogen fermentation processes, because they suppress the methanogenic bacteria that requires relatively longer times to grow as compared to acidogenic bacteria (Liu et al., 2008). For example, methanogens are known to have slow specific growth rates of 0.0167-0.02 h^{-1} whereas acidogens are reported to have a relatively higher specific growth rate of 0.172 h^{-1} (Khanna and Das, 2013).

Kim et al. (2004) reported that short HRTs below 72 hours increase the biohydrogen efficiency. Several studies on biohydrogen fermentation processes have indicated that the pH and HRT are joint parameters (Lee et al., 2010; Liu et al., 2008; Shin and Youn, 2005); since it has been shown that short HRTs result in low pH. In addition, both of these parameters have been viewed as effective in inhibition of biohydrogen-consuming bacteria (Oh et al., 2004). HRT controls microbial growth and hence this process parameter must be greater than the maximum growth rate of bacteria to prevent biomass washout (Hallenbeck and Ghosh, 2009).

2.13.4 Organic Loading Rate

Organic Loading rate (OLR) is a measure of biological conversion capacity of the anaerobic digestion process (Morimoto et al., 2004). The OLR affects various fermentation conditions, such as the production of VFAs, COD removal efficiency, pH, as well as variations in the composition of the active biomass, with consequent modifications of the associated metabolic

pathways (De Gionnis et al., 2013). However, OLR is also affected by various parameters such as the type of substrates, temperature, and source of inoculum used. Several authors investigated the effect of OLR on biohydrogen production. For example, Shin and Youn (2005) observed that increasing OLR up to 8 g VS/L/d while maintaining long HRT of 5 days enhanced the production of biohydrogen. Hong and Haiyun (2010) maximized the production of biohydrogen when the OLR was increased from 4 to 8 g VSS/L/d at long HRT of 8.92 days from food waste. A maximum biohydrogen production rate of 5.4 L H_2/d was reported at OLR of 29 g COD /L d and 110 g TVS/ L d, respectively by Tawfik and El-Qelish (2012) and Zahedi et al. (2012).

2.13.5 Bioreactor configuration

Different bioreactor configurations have been used in biohydrogen production studies. The size of these bioreactors varies from small-scale (100-500 mL) to semi-pilot scale (2-10 L) or pilot-scale (>20 L) and are operated under batch, semi-continuous or continuous conditions (Saka and Kumar, 2010; Show et al., 2011). In an industrial-scale process; continuous fermentation processes are recommended for evaluation of various aspects such as monitoring the fermentation conditions, production and yield, and practical engineering aspects (Ismail et al., 2009). Examples of bioreactor configurations used in biohydrogen production include:

- Continuous stirred tank reactors (CSTRs)
- Upflow anaerobic sludge blanket reactors (UASBRs)
- Anaerobic fluidized bed reactors (AFBRs)
- Anaerobic sequencing batch reactors (ASBRs)
- Membrane reactors (MRs)

Amongst these reactors, CSTRs are widely used in biohydrogen fermentation processes (Gomez et al., 2006; Kim et al., 2011; Nandi and Sengupta, 1998) because they offer effective homogenous mixing patterns. Furthermore, CSTRs ensure good substrate-microbe contact as well (Show et al., 2011). The reactors could reach steady-state and exhibit high efficiency and stable performance when the operational conditions are optimized (Show et al., 2011). The setback for the application of CSTRs in continuous biohydrogen fermentation is biomass washout. Therefore, to ensure stable biohydrogen production processes, the use of reactor systems with immobilized cells, such as anaerobic fluidized bed reactors (AFBRs) and upflow anaerobic sludge blanket reactors (UASBRs), has been proposed (Singh and Wahid, 2014). Immobilized reactors offer many advantages such as higher biomass density, improved operation stability, easier separation of solids and liquids, and reduced risk of contamination (Abreu et al., 2012; Hung et al., 2007; Singh and Wahid, 2014; Temudo et al., 2007; Yasin et al., 2011). The biohydrogen-producing operational setpoint parameters reported in various studies of biohydrogen fermentation processes are summarized in Table 2.5.

Table 2.5: Dark fermentative biohydrogen-producing operational setpoint parameters reported in literature.

Substrate	pH	Temp (^oC)	HRT (h)	OLR	Reactor	H_2 yield	Reference
Molasses	5.5	35	12	80 g COD/L/d	CSTR	131. 2 H_2/kg COD	Lay et al. (2012)
Pig slurry	-	70	24	33.2 g VS/L/d	CSTR	0.42 H_2/kg COD	Kotsopoulos et al. (2009)
Cheese whey	5.9	37	6	139 g lactose/L/d	UASB	18.8 g H_2/kg COD	Davila-Vasquez et al. (2008)
Coffee WW	5.5	35	12	8.3 g H_2/kg COD	UASB	8.3 g H_2/kg COD	Yang et al. (2006)
Synthetic WW	4	35	1	13 g glucose/L/d	Fluidized bed	12.5 g H_2/kg COD	Zhang et al. (2008)
Starch WW	6.5	37	12	-	CSTR	1.28 mol H_2/mol glucose	Chen et al. (2008b)
Food waste	6.5	35	-	-	Batch	593 ml H_2/g carbohydrate	Nazlina et al. (2009)
Distillery waste	4-7	25-55	-	-	Batch	3.35 mol H_2/mol glucose	Kamalaskar et al. (2010)
Food waste	5.4-5.7	30	-	-	UASB	11.1 L H_2/L/day	Lee et al. (2010)
Citric acid WW	6.8-7.2	35-38	6	38.4 g COD/L/d	UASB	0.84 mol H_2/mol hexose	Yang et al. (2006)
Swine manure	5	35	16	-	SFF	0.00187 g H_2/TVS	Zhu et al. (2009)
Apple pomace	7	37	-	-	Batch	134.04 ml H_2/g TS	Wang et al. (2010a)
Waste bread	≥ 4	30	6	-	CSTR	7.4 L H_2/L/d	Wei et al. (2016)
OFSMW	6-7	37	24	-	Batch	61 ml H_2/g TVS	Shah et al. (2016)
Food waste	5.5	55, 70	240	14 kg/COD/m^3/d	Batch	70.7 ml H_2/g VS	Algapani et al. (2016)
OFSMW	7.9	30.29	60	-	Batch	246.93 ml H_2/g TVS	Sekoai and Gueguim Kana (2014b)
Potato waste	11	35	72	-	Batch	1.73 mol H_2/mol glucose	Faloye et al. (2014)
Sugarcane	6	37	68	-	Batch	248.05 ml H_2/g TVS	Moodley and Gueguim Kana (2015)
Sorghum	4.7	35	4-24	-	CSTR	10.4 L H_2/kg sorghum	Antonopoulou et al. (2008)
Wheat	7	37	-	-	Batch	1.46 mol H_2/mol glucose	Sagnak and Kargi (2011)

-: data not available, OFSMW: Organic Fraction of Solid Municipal Waste, CSTR: Continuous Stirred Tank Reactor, OLR: Organic Loading Rate, UASB: Upflow Anaerobic Sludge Blanket Reactor, SFF: Semi-Continuously-Fed Fermenter, WW: Wastewater.

2.14 Microbiology of dark fermentation process

2.14.1 The dark fermentative biohydrogen-producing microorganisms

Dark fermentative bioprocesses are conducted using diverse microorganisms which are used as pure cultures. Many studies documented in literature used mixed cultures (Abreu et al., 2012; Lay et al., 2012; Lin et al., 2011; Ozmihci and Kargi, 2010; Wang et al., 2010b). The production of biohydrogen from mixed cultures is more beneficial as compared to pure cultures due to the following reasons: (i) there is minimum sterility required, (ii) the high level of microbial diversity increases the conversion efficiency, (iii) there is microbial synergism, (iv) it favours continuous bioprocesses, and (v) use diverse substrates (Kleerebezem and van Loosdrecht, 2007; Temudo et al., 2007). These organisms are isolated from diverse environments such as soils, composts, sewage sludge, and wastewaters.

Microbial community analysis of various biohydrogen-producing activated systems showed that members of the genus *Clostridium* are dominant and active biohydrogen-producers (Das and Veziroglu, 2001; Fang et al., 2002; Hung et al., 2007; Wang and Wan, 2008). They are Gram positive, spore-forming, and are rod-shaped obligate anaerobes. They also utilize a variety of substrates which is of great interest for biohydrogen process development (Madigan et al., 1997; Wang et al., 2008). Their proportion is reported to be more than 60% of total bacterial populations after pretreatments (Pan et al., 2008). This is likely attributed to the heat resistance of spores (Fang et al., 2006; Wang et al., 2008). Many studies of biohydrogen production processes have used *Clostridium* species, including includes *C. butyricum* (Yokoi et al., 2002), *C. beijerinckii* KCTC 1785 (Kim et al., 2008), *C. bifermentans* (Wang et al., 2003), and *C. tyrobutyricum* ATCC 25755 (Liu et al., 2006). Lin et al. (2007) studied the effect of four clostridial strains *C. acetobutylicum* M121, *C.*

butyricum ATCC19398, *C. tyrobutyricum* FYa102, and *C. beijerinckii* L9, respectively and obtained a high yield of 2.81 mol H_2/mol glucose.

Members of the genus *Enterobacter* have also been reported to be effective for biohydrogen production (Khanna et al., 2011; Kumar and Das, 2000; Ozmihci and Kargi, 2010; Tanisho et al., 1987; Yokoi et al., 1995). These species are facultative anaerobes, Gram negative and rod-shaped organisms. They produce low biohydrogen concentrations as compared to *Clostridium* species (Tenca et al., 2011). Kumar and Das (2000) enhanced the production of biohydrogen using *Enterobacter cloacae* IIT-BT 08 and achieved a maximum yield of 2.2 mol H_2/mol glucose. Facultative anaerobes such as *Bacillus* species are reported as well (Liu and Wang, 2012; Manikkandan et al., 2009; Meher Kotay and Das, 2008). Other biohydrogen-producing bacteria include *Pseudomonas* sp., *Actinomyces* sp., *Streptococcus* sp., *Klebsiella* sp. and *Escherichia coli* (Hung et al., 2007; Oh et al., 2003). In pure cultures, metabolic pathways are easily detected due to the reduced diversity of the biomass. Moreover, studies employing pure cultures can reveal important information regarding conditions that promote high hydrogen yield and production rate (Elsharnouby et al., 2013). Nonetheless, using pure cultures has its own limitations such as strict sterilization procedures and the selectivity of substrates (Hawkes et al., 2002).

Microbial conversion of glucose (carbon source) by biohydrogen-producing *Clostridium* species is associated with two metabolic pathways as shown in Figure 2.6. The first pathway involves the conversion of pyruvate to acetyl-CoA and CO_2 through pyruvate ferredoxin oxidoreductase i.e. (1), with production of reduced ferredoxin (Fd). Molecular hydrogen is generated from the reduced ferredoxin through hydrogenase enzyme activity. The NADH produced from glycolysis by the enzyme NADH-ferredoxin oxidoreductase is re-oxidized in the second pathway to produce reduced ferredoxin i.e. (2) (Vardar-Schara et al., 2008), which is then used by the hydrogenase enzyme to produce hydrogen. Biohydrogen-producing

Clostridium species can stoichiometrically produce 2 or 4 mols using either butyrate or acetate pathway. However, experimental biohydrogen yields are low due to formation of other fermentative by-products. Low production yields are produced by butyrate pathways because it exhibits some inhibitory effects on biohydrogen-producing bioprocesses (Berrios-Rivera et al., 2000; Chin et al., 2003). Another limitation is that it uses more NADH which is one of the most important enzymatic co-factors during biohydrogen production (Kumar et al., 2001). Figure 2.7 shows the diverse bacterial groups that are involved in dark fermentation process. These microorganisms are classified based on their sensitivity to temperature i.e. mesophiles, thermophiles, and extreme thermophiles; and oxygen i.e. obligate anaerobes, facultative anaerobes, and aerobes. Mesophiles are cultured at moderate temperatures while thermophiles and extreme thermophiles require elevated temperatures for their growth. Meanwhile, obligate anaerobes grow in the absence of oxygen, while strict aerobes and facultative anaerobes grow in oxygen containing medium (Das, 2009).

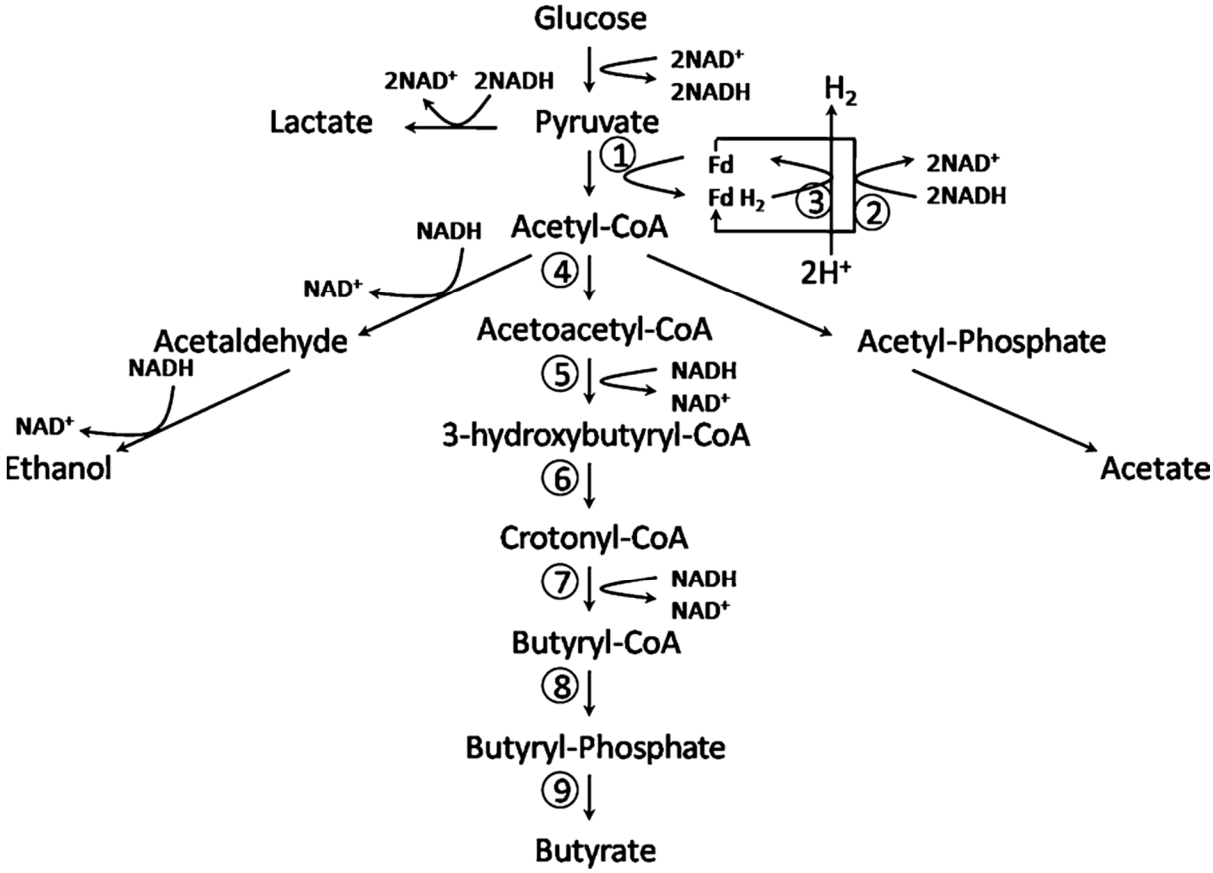


Figure 2.6: Metabolic pathways of biohydrogen-producing clostridia and enzymes involved in its production, (1) pyruvate-ferredoxin oxidoreductase; (2) NADH-ferredoxin oxidoreductase, (3) hydrogenase, (4) acetyl-CoA acetyltransferase, (5) β-hydroxybutyryl-CoA dehydrogenase, (6) 3-hydroxybutyryl-CoA dehydratase, (7) butyryl-CoA dehydrogenase, (8) phosphotransbutyrylase; (9) butyrate kinase (Cai et al., 2011).

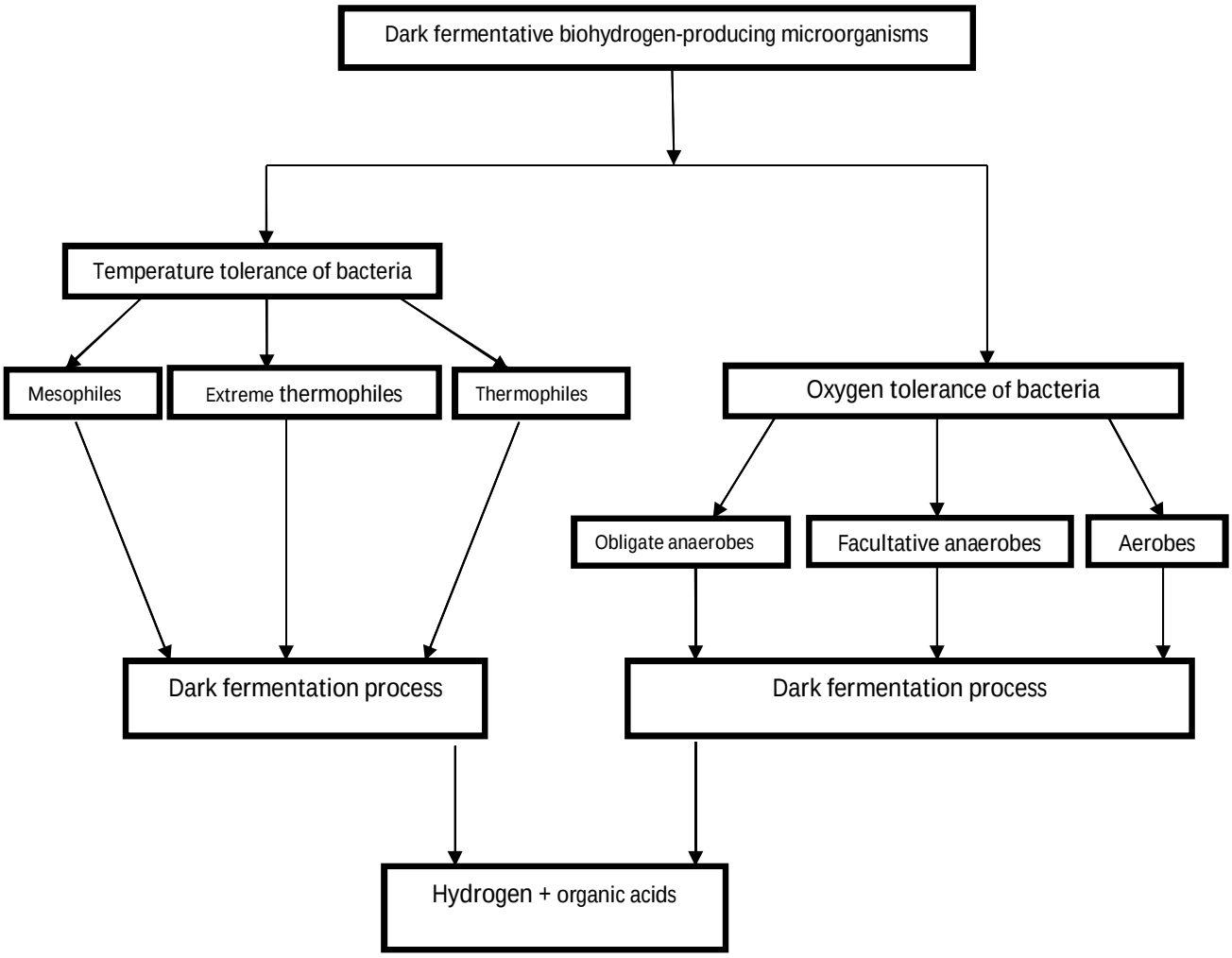


Figure 2.7: Schematic representation of dark fermentative biohydrogen-producing bacteria (Chandrasekhar et al., 2015).

2.15 Dark fermentation as a process

2.15.1 Advantages, limitations, and potential

Dark fermentation has been repeatedly highlighted as a promising renewable source of energy, and has received considerable attention in recent years due to its social, economic and environmental merits (Meher Kotay and Das, 2008). In addition, it provides an avenue for effective disposal and beneficiation of biowaste materials such as agricultural, municipal and industrial effluents (Nath and Das, 2004; Pandu and Joseph, 2012). Nonetheless, the realization of a dark fermentation driven economy has been hindered by its low production yield. A theoretical analysis reveals that the maximum yield by *Clostridium* species on glucose is 4 mol H_2/mol glucose when acetate is produced (see Equation 2.1) or 2 mol H_2/mol glucose when butyrate is produced as shown in Equation 2.2 (Saleno et al., 2006).

Acetate: $C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$ (2.1)

Butyrate: $C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$ (2.2)

The accomplishment of higher bioprocess yields is still a crucial research issue in dark fermentation bioprocess technology (Sekoai et al., 2016). Currently, microbial dark fermentation processes can only produce 2-3 mol H_2/mol glucose, resulting in 80-90% of initial chemical oxygen demand (COD) remaining in solution in the form of various volatile organic acids and solvents (Liu et al., 2010; Saleno et al., 2006), this phenomenon is referred to as the dark fermentation “process barrier”. There are several “process barriers” that affect the overall dark fermentative biohydrogen yields; these include hydrogenotrophic methanogens, homoacetogens, nitrate-reducing bacteria, sulphate-reducing bacteria, organic acids, and other end-products. Thus, to improve the process economics of dark fermentation from biowaste; various strategies such as metabolic engineering, two-stage fermentation processes, application of optimization tools, and pretreatment methods are pivotal in dark

fermentation process technology (these strategies are discussed in section 2.17.2). In addition, more nutrient-rich substrates need to be exploited for its process development. The utilization of biowaste effluents for dark fermentation processes is scantily reported in most African countries. Thus, this impedes initiatives for development of renewable and sustainable energy production within the continent. In addition, as a response to the Millennium Development Goal (MDG), devising better waste management options could promote environmental security and sustainability in the continent. A report from the United Nations has shown that proper waste management facilities are still lacking in Africa (United Nations, 2009). Hence, there is widespread dumping of waste in water bodies and landfills which in turn aggravates the challenges of sanitation. Other contributing factors include urbanization which is said to be on the rise in Africa i.e. Africa is estimated to have an urban growth of 3.5% per annum which is the highest in the world (United Nations, 2009). Thus, several practices have been proposed in several countries to combat this challenge. Among these, conversion of waste to energy is being implemented as the continent faces the energy crisis and climate change.

Dark fermentative biohydrogen production from biowaste effluents has the potential to become a cost competitive energy generating process owing to their nutritional composition and accessibility. Furthermore, South Africa will increasingly generate more waste due to the high level of urbanization and industrialization as emphasized earlier. Therefore, the production of biohydrogen from these waste materials will make a significant contribution to the generation of clean fuel, mitigation of environmental pollution, and reduce their disposal costs. As the maximum theoretical yield of biohydrogen production on pure glucose substrate is low (4 mol H_2/mol glucose), dark fermentation from these waste effluents may enhance the overall biohydrogen production rates and yields.

2.15.2 Synergy between dark fermentation and other biohydrogen production processes

The need for hybrid processes is highly emphasized in dark fermentation studies in order to improve the overall biohydrogen conversion efficiency from biowaste substrates. The residual/medium from dark fermentation process is used as a substrate in other biohydrogen-producing processes such as photo-fermentation, Microbial Fuel Cells (MFCs), Microbial Electrolysis Cells (MECs), and biogas production as indicated in Table 2.6 and Figure 2.8, respectively. Chen et al. (2008a) reported a COD removal efficiency of 90% in hybrid processes of dark and photo-fermentation process. Lalaurette et al. (2009) also reported a 90% COD removal efficiency in a two-stage process of dark fermentation and MEC. Meanwhile, Massanet-Nicolau et al. (2015) reported a biohydrogen increase of 13.4% in a two-stage process of biohydrogen and biomethane production. Hybrid processes of dark and photo-fermentation are encouraged due to high conversion efficiency. Photo-fermentative biohydrogen-producing bacteria can utilize the organic acids (acetic, butyric, propionic, valeric acid) found in dark fermentation medium (Equation 2.3) for further biohydrogen conversion. For example, 8 mols of biohydrogen can be generated from acetate-rich effluents as shown in Equation 2.4 (Bala Amutha and Murugesan, 2011). However, the process of photo-fermentation has its own limitations such as the need for an (i) external light source, (ii) maintenance of photo-fermentative bacteria, (iii) high risks of contamination, and (iv) the process will be expensive at large-scale (Khanna and Das, 2013; Ozmihci and Kargi, 2010; Uyar et al., 2015).

Dark fermentation (Stage1): $C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$ (2.3)

Photo-fermentation (Stage 2): $2CH_3COOH + 4H_2O \rightarrow 8H_2 + 4CO_2$ (2.4)

Other biological hydrogen production processes (e.g. direct and indirect biophotolysis) are presented in Table 2.7. Among these processes, dark fermentation is highly favoured due to

its several process advantages such as, (i) utilization of diverse carbon sources including the treatment of waste materials, (ii) utilization of diverse microorganisms which are found in sludge, soil samples, industrial and municipal sites, (iii) this process can be conducted at ambient temperature and pressure, (iv) the levels of contamination are low, (v) it can be integrated with other biohydrogen production processes as shown in Figure 2.8. Nonetheless, this process has its own constrains such as low biohydrogen yields as a result of metabolites and thermodynamic limitations. Thus, optimization strategies are highly essential in dark fermentative biohydrogen process development, and they are elaborated in section 2.17.2.

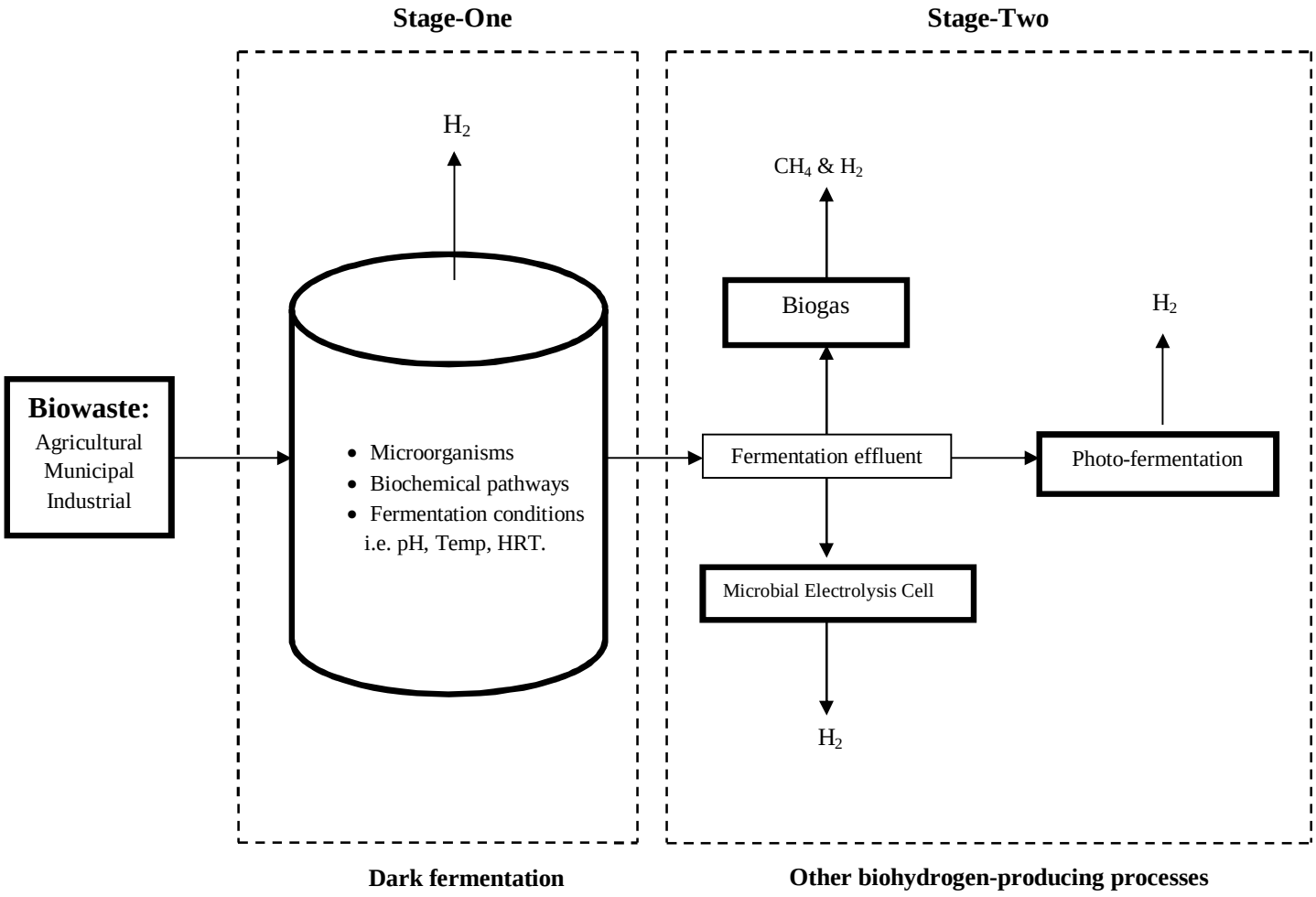


Figure 2.8: Dark fermentation process integrated with other biohydrogen-producing processes (Show et al., 2011).

Table 2.6: Two-stage processes involving dark fermentation process and other biohydrogen-producing processes.

First stage	Second stage	Microorganism for 2nd stage	Fermentation conditions	COD recovery (%)	H_2 yield	Reference
Dark-fermentation	Photo-fermentation	*Rhodopseudomonas palustris* WP3-5	32 ^oC, pH 7.1, 100 rpm, light intensity of ca. 95 W/m^2	72	10.02 mol H_2/mol sucrose	Chen et al. (2008a)
Dark-fermentation	Biomethane production	Unpretreated anaerobic sludge	35 ^oC, pH 7.5, OLR of 20-35 kg COD/m^3 d	98	-	Kisielewska et al. (2013)
Dark-fermentation	Microbial Fuel Cell	Mixed sludge	29 ^oC, pH 7.0, OLR of 0.99-3.13 kg COD/m^3 day	84.6	-	Mohanakrishna et al. (2010)
Dark-fermentation	Microbial Electrolysis Cell	Domestic wastewater	25 ^oC, pH 7.0, voltage of 25 mV	23	33.2 mmol H_2/g COD	Wang et al. (2011)

-: data not available.

Table 2.7: Biological hydrogen-producing processes with their advantages and limitations (Khanna and Das, 2013).

Biohydrogen process	General reaction of the process	Advantages	Process limitations
Dark fermentation process	$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$	Uses diverse substrates Uses diverse bacteria Minimum sterility required It's a cost-effective process	Low H_2 yields Low substrate conversion efficiency Metabolites inhibits H_2 production Gas mixtures containing CO_2 requires separation
Photo-fermentation	$CH_3COOH + 2H_2O + Light \rightarrow 4H_2 + 2CO_2$	Uses various carbon source Uses diverse light source	Requirement for an external light source Light conversion is low Low H_2 yields due to poor light source
Direct biophotolysis	$2H_2O + light \rightarrow 2H_2 + O_2$	Uses diverse waste materials	Requirement for an external light source Low light conversion Hydrogenase enzyme sensitive to O_2
Indirect biophotolysis	$12H_2O + light \rightarrow 12H_2 + 6O_2$	Can produce H_2 from water Can fix N_2 from the atmosphere	Low H_2 yields due usage of hydrogenase Requirement for an external light source Enzyme inhibition by O_2

2.16 Economic evaluation of dark fermentation from biowaste effluents

Despite the extensive research that has been carried out over the past decade, few researchers have evaluated the economic potential of industrial-scale biohydrogen production processes. Classen et al. (2000) conducted a cost evaluation on a biohydrogen-producing dark-fermenter (total volume = 95 000 L) and a photo-fermenter (total volume = 300 000 L). The production capacity for these vessels was 39 kg H_2/h, and the overall costs were estimated at US \$3.65 kg^{-1} H_2. However the costs of biomass, construction, and labour were not included. A biohydrogen production rate of 425 000 L H_2 h^{-1} was postulated from the process and this corresponded to an energy equivalent of 5.4 GJ h^{-1} (Classen et al., 2000). Meanwhile, Benemann (2000) conducted an initial cost analysis for algal biohydrogen production system. The reactor had a capacity of 25 694 kg H_2/day which corresponded to 3600 GJ/day. The costs for the algal reactor were projected at US \$43 million, whereas the annual operating costs were US \$12 million/year. In this evaluation, the capital costs accounted for 90% the overall costs (Benemann, 2000). de Vrije and Classen (2003) also conducted the cost analysis of biohydrogen fermentation processes using lignocellulose materials. The plant capacity was 910 kg H_2 day^{-1} and consisted of a 95, 000 litre thermo-bioreactor for dark fermentation which was coupled to a 300 000 L photo-fermenter. The production costs were estimated at US \$3 dollar per kg H_2, without taking into accounts the cost of hydrolysis. Therefore, all the above cost analyses are based on assumptions and aimed to assess the economic feasibility of the process on a commercial-scale. Nonetheless, more R&D should be invested in dark fermentative biohydrogen process because this technology is more expensive as compared to other fuel options due to its process complexities. This implies that many technical and engineering challenges need to be tackled before this technology can be implemented on an industrial-scale.

2.17 Challenges and the way forward for the dark fermentation process

2.17.1 Technical challenges facing dark fermentation scale-up studies from biowaste

A critical challenge facing scale-up studies of biohydrogen production from biowaste is the low biohydrogen conversion efficiency (Das and Veziroglu, 2001; Guo et al., 2010). This is attributed to the accumulation of biohydrogen inhibiting reactions such as solventogenesis and methanogenesis during dark fermentation process (Khanal et al., 2004; Sekoai and Gueguim Kana, 2014a, b). Biohydrogen production intermediates such as volatile fatty acids, propionate, ethanol, carbon dioxide, and biohydrogen-consuming bacteria (like homoacetogens and methanogens), lower the overall biohydrogen yield (Lay et al., 1999). Hitherto, the maximum biohydrogen yield reported in literature is 2.3-2.91 mol H_2/mol glucose (Masset et al., 2012; Wong et al., 2014) from pure strain of *Clostridium* species. This process is still not commercially viable. Moreover, a study conducted by Sekoai and Gueguim Kana (2014) highlighted some limitations with the utilization of biowaste effluents for biohydrogen fermentation processes: (i) these feedstocks consists of many compounds and thus some may have inhibitory effects on dark fermentation pathways; (ii) these effluents are usually dispersed, and this might escalate their collection costs (iii) the lignin structure of biowaste materials is hard to penetrate, thus pretreatment strategies such as mechanical, physical, chemical and biological procedures are often adopted to break down the lignocellulose content thereby enhancing the release of soluble sugars and accessibility to microorganisms during fermentation. However, these pretreatments methods are energy-intensive and expensive (Esteghlalian et al., 2002; Zheng et al., 2009).

2.17.2 Strategies for optimization of dark fermentation process yields from biowaste

Several optimization strategies have been proposed in dark fermentative biohydrogen production studies for enhancing its conversion efficiency from biowaste feedstocks. These strategies are discussed below:

- Glucose is an ideal substrate in dark fermentation process but it is too costly to support its large-scale production. Thus, utilization of nutrient-rich biowaste substrates is a viable approach to overcome some of the economic constrains of dark fermentative biohydrogen process development.
- Cost-effective pretreatments of biowaste materials are necessary to improve the biohydrogen conversion efficiency because some of these substrates contain high amounts of lignocellulose.
- The use of optimization tools such as response surface methodology (RSM) and Artificial Neural Network (ANN) may significantly improve the overall yields because these statistical methods determine the synergistic optimum parameters that are favourable for biohydrogen fermentation processes (Sekoai and Gueguim Kana, 2014a).
- There is a need for bioreactor designs with high level of parallelization coupled with online monitoring devices for detecting the critical fermentation conditions during biohydrogen processes. The development of micro-sensors in bioreactors is essential in order to provide real-time and reliable bioprocess data and also to determine suitable parameter setpoints for maximum biohydrogen production (Sekoai and Gueguim Kana, 2014a).
- Integration of hybrid bioprocesses is vital in order to enhance the overall biohydrogen conversion efficiency. These include (i) dark fermentation and Microbial Electrolysis Cells (MECs), (ii) dark- and photo-fermentation processes, and (iii) dark fermentation and biomethane production (Argun et al., 2008).
- Cost-effective pretreatment methods of the inoculum are necessary for the growth of dark fermentative biohydrogen-producing bacteria (e.g. *Clostridium* sp., *Bacillus* sp.) while suppressing biohydrogen-consuming microorganisms.

- Utilization of co-substrates has been shown to improve the dark fermentation process yields. For instance, Zhu et al. (2008) observed that the combination of the substrates (food waste + primary sludge + waste activated sludge) enhanced the overall yields as compared to individual substrates. Meanwhile, Sekoai and Gueguim Kana (2013) reported a 3.8% in biohydrogen increase from the organic fraction of municipal solid waste comprising of apple waste, orange waste, cabbage waste, potato waste, bread waste, and paper waste respectively. Therefore, these wastes provide a desirable carbon and nitrogen (C/N) ratio for biohydrogen-producing bacteria.
- Metabolic engineering has also gained much attention over the past few years and it could potentially improve the biohydrogen yields. Efforts have been focusing on redirection, identification and engineering of oxygen tolerant hydrogenases (Sinha and Pandey, 2011). Studies have also focused on metabolic pathways to regulate the biohydrogen-producing reactions and biohydrogen-producing microorganisms. However, some reports in literature have highlighted a need for an extension of substrates in metabolic studies of biohydrogen-producing bacteria because these organisms are fastidious (Eroglu et al., 2009).
- Another technology that is gaining increasing prominence in biohydrogen process development is cell immobilization. It offers several advantages such as high metabolic activity; increases cell density, easier handling, better solid/liquid separation efficiency, and better operational stability (Bardi and Kountinas, 1994; Wu et al., 2006). It is used in various reactor prototypes such as continuous stirred tank reactors (Fang et al., 2002), fluidized bed reactors (Lin et al., 2006), carrier induced granular sludge beds (Argun et al., 2008), up-flow anaerobic sludge bed reactors (Lee et al., 2004), and trickling biofilters (Roy et al., 2014). The immobilization methods include granulation (Chang and Lin, 2009), biofilm formation (Show et al., 2011), gel

entrapment (Ivanova et al., 2009), ceramics or glass beads (Perego et al., 1989), cellulosic materials (Goncalves et al., 1992), and polyacrylamide gels (Hsu et al., 1980; Liu et al., 2010). Furthermore, a review article that was published in "*Critical Reviews in Biotechnology*" demonstrated that immobilized microorganisms possess the following advantages in biohydrogen production (Sekoai et al., 2017):

- Increases the biohydrogen yields during dark fermentation process.
- Withstands the harsh fermentation conditions i.e. solvents, pH, and toxic metals.
- Has the potential to increase the substrate conversion efficiency.
- Minimize the levels of microbial contamination.
- Minimize the need for separation and filtration steps.
- Prolongs the biohydrogen-producing acidogenic process.
- Protects the microbes against shear stress caused by stirring.
- Possible reusability of microorganisms.

Cell immobilization will also be explored in this study towards the enhancement of biohydrogen yields during dark fermentation experiments. Some of the abovementioned optimization strategies are summarized in Figure 2.9.

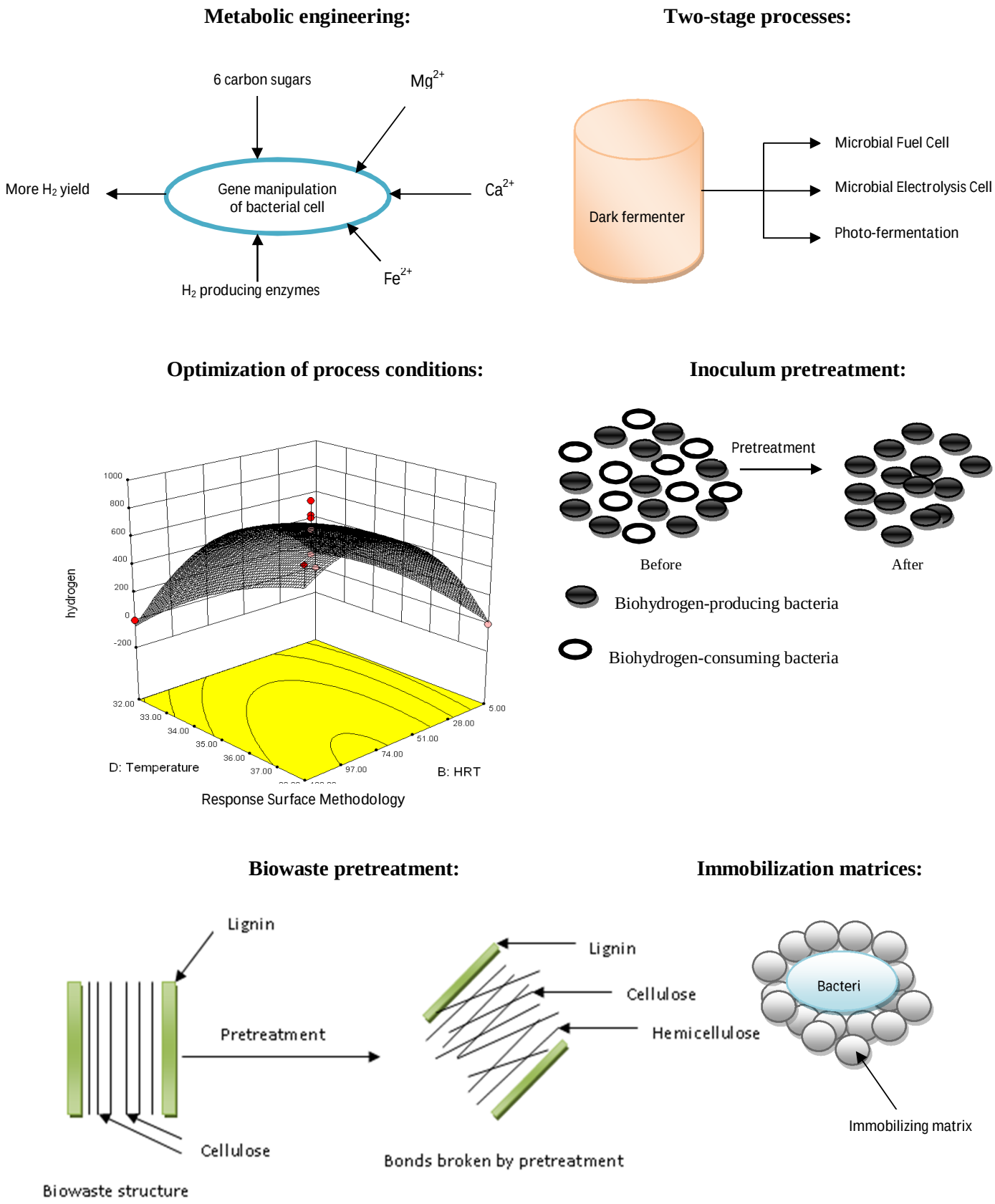


Figure 2.9: Optimization strategies for dark fermentative biohydrogen production.

2.18 Summary

Dark fermentative biohydrogen production from biowaste effluents demonstrates the possibilities of generating alternative and sustainable energy fuels that are environmentally friendly and reliable in South Africa. The availability of biohydrogen as a clean alternative source of energy could pave the way to meeting the country's escalating energy demands. Furthermore, the use of biowaste which is abundantly present in South Africa for biohydrogen production, will significantly improve the process economics of the process. However, to fully realize the commercialization of biohydrogen production in South Africa and the rest of the world, it is imperative for both the government and private sector to invest enormously on technological development and technical expertise pertaining to biohydrogen fermentation processes. The economic analysis of dark fermentation process shows that the unit price of biohydrogen production will be more expensive at industrial-scale as compared to energy derived from fossil fuels due to its process complexities such as low conversion efficiency, accumulation of by-products that compete with biohydrogen-producing pathways, the need for optimum bioreactor designs, the need for biohydrogen purification methods, and the requirements for hydrogen storage systems. Nonetheless, biohydrogen is still a preferred energy fuel when taking into account the adverse effects of climate change, dwindling fossil reserves, and escalating energy prices.

Some of the challenges (e.g. low biohydrogen yield) highlighted in this chapter will be addressed in this dissertation. For instance, various biohydrogen enhancement methods such as the utilization of nutrient-rich feedstocks, parametric optimization, cell immobilization, the use of metal ions, and nitrogen gas sparging, will be explored in this study. The work discussed in this chapter has resulted in two review articles that were published in "*International Journal of Renewable Energy Research*" and "*Biofuel Research Journal*".

References

Abreu, A.A., Karakashev, D., Angelidaki, I., Sousa, D.Z., and Alves, M.M. (2012). Biohydrogen production from arabinose and glucose using extreme thermophilic anaerobic mixed cultures. Biotechnology for Biofuels 5, 1-12.

Algapani, D.E., Qiao, W., Su, M., di Pumpo, F., Wandera, S.M., and Adani, F. (2016). Bio-hydrolysis and bio-hydrogen production from food waste by thermophilic and hyperthermophilic anaerobic process. Bioresource Technology 216, 768–777.

Angeriz-Campoy, R., Álvarez-Gallego, C.J., and Romero-García, L.I. (2015). Thermophilic anaerobic co-digestion of organic fraction of municipal solid waste (OFMSW) with food waste (FW): enhancement of bio-hydrogen production. Bioresource Technology 194, 291–296.

Antonopoulou, G., Gavala, H., Skiadas, I., Angelopoulos, K., and Lyberatos, G. (2008). Biofuels generation from sweet sorghum: Fermentative hydrogen production and anaerobic digestion of the remaining biomass. Bioresource Technology 99, 110-119.

Argun, H., and Dao, S. (2016). Hydrogen gas production from waste peach pulp by dark fermentation and electrohydrolysis. International Journal of Hydrogen Energy 41, 11568-11576.

Argun, H., Kargi, F., and Kapdan, I.K. (2008). Light fermentation of dark fermentation effluent for bio-hydrogen production by different *Rhodobacter* species at different initial volatile fatty acid (VFA) concentration. International Journal of Hydrogen Energy 33, 7405–7412.

Arooj, M.F., Han, S.K., and Kim, S.H. (2008). Continuous biohydrogen production in a CSTR using starch as a substrate. International Journal of Hydrogen Energy 33, 3289–3294.

Bala Amutha, K., and Murugesan, A.G. (2011). Biological hydrogen production by the algal biomass *Chorella vulgaris* MSU 01 strain isolated from pond sediment. Bioresource Technology 102, 194-199.

Banks, D., and Schaeffler, J. (2006). The potential contribution of renewable energy in South Africa. Available at: http://earthlife.org.za/www/wp-content/uploads/2009/04/potential-of-re-in-sa-feb06.pdf. (Accessed 18 March 2016).

Bardi, E., and Kountinas, A.A. (1994). Immobilization of yeast on delignified cellulose material for room and low-temperature wine-making. Journal of Agriculture and Food Chemistry 42, 221–226.

Barry, M.L., Steyn, H., and Brent, A. (2011). Selection of renewable energy technologies for Africa: Eight case studies in Rwanda, Tanzania and Malawi. Renewable Energy 36, 2845-2852.

Benemann, J.R. (2000). Hydrogen production by microalgae. Journal of Applied Phycology 12, 291-300.

Berrios-Rivera, S.J., Yang, Y.T., Bennett, G.N., and San, K.Y. (2000). Effect of glucose analog supplementation on metabolic flux distribution in anaerobic chemostat cultures of *Escherichia coli*. Metabolic Engineering 2, 149–154.

Bio2Watt, (2016). Available at: http://www.bio2watt.com/bio2watt%E2%80%99s-bronkhorstspruit-biogas-plant-(pty)-ltd.html. (Accessed 18 March 2016).

Blanchard, R., Richardson, M.D., O'Farrell, P.J., and von Maltitz, G.P. (2011). Biofuels and biodiversity in South Africa. South African Journal of Science 107, 1-8.

Boboescu, I.Z., Gherman, V.D., Lakatos, G., Pap, B., and Mariti, T.B.G. (2016). Surpassing the current limitations of biohydrogen production systems: The case for a novel approach. Bioresource Technology 204, 192-201.

BP, (2013). Statistical Review of World Energy. Available at: http://www.bp.com/content/dam/bp/pdf/statistical-review/statisticalreviewofworldenergy2013.pdf . (Accessed 18 March 2016).

Bredell, J.H. (1987). South African coal resources explained and analysed. Geological Survey of South Africa, 1-39.

Brodski, S. (2009). South Africa's REFIT Programme Starts to Take. Dewey & LeBoeuf for Energy Indaba. Available at: http://www.energyafricaexpo.com/newsletter/October09.html. (Accessed 18 March 2016).

Broomfield, M., Davies, J., and Harrison, R. (2004). Review of Environmental and Health Effects of Management of Municipal Solid Waste. Department for Environment, Food and Rural Affairs. London, United Kingdom.

Buys, W.Y. (2015). Investigating the effect of wine and distillery wastewater on the efficacy of an upflow anaerobic sludge blanket (UASB) and enhancing biomass immobilisation by the addition of magnetisable foam glass particles. MSc Thesis. Stellenbosch University, South Africa.

Cai, G., Jin, B., Saint, C., and Monis, P. (2011). Genetic manipulation of butyrate formation pathways in *Clostridium butyricum*. Journal of Biotechnology 155, 269-274.

Cappai, G., De Gioannis, G., and Giordano, G. (2009). Energy and material recovery through combined hydrogen-methane production and final composting of different solid and liquid waste. Proceedings of 12th International Waste Management and Landfill Symposium. Margherita di Pula, Italy.

Cavinato, C., Giuliano, A., Bolzonella, D., Pavan, P., and Cecchi, F. (2015). Bio-hythane production from food waste by dark fermentation coupled with anaerobic digestion

process: a long-term pilot scale experience. International Journal of Hydrogen Energy 37, 11549–11555.

Chandrasekhar, K., Lee, Y.J., and Lee, D.W. (2015). Biohydrogen Production: Strategies to Improve Effiency through Microbial Routes. International Journal of Molecular Science 16, 8266-8293.

Chang, F.Y., and Lin, C.Y. (2009). Biohydrogen production using an up-flow anaerobic sludge blanket reactor. International Journal of Hydrogen Energy 29, 33–39.

Chen, C., Lin, C., and Chang, J. (2001). Kinetics of hydrogen production with continuous anaerobic cultures utilizing sucrose as the limiting substrate. Applied Microbiology and Biotechnology 57, 56-64.

Chen, C.Y., Yang, M.H., Yeh, K.L., Liu, C.H., and Chang, J.S. (2008a). Biohydrogen production using sequential two-stage dark and photo fermentation processes. International Journal of Hydrogen Energy 33, 4755-4762.

Chen, S.D., Lee, K.S., Lo, Y.C., Chen, W.M., Wu, J.F., Lin, C.Y., and Chang, J.S. (2008b). Batch and continuous biohydrogen production from starch hydrolysate by *Clostridium* species. International Journal of Hydrogen Energy 33, 1803–1812

Cheng, X.Y., and Liu, C.Z. (2011). Hydrogen production via thermophilic fermentation of cornstalk by *Clostridium thermocellum*. Energy Fuels 25, 1714-1720.

Chin, H., Chen, Z., and Chou, C.P. (2003). Fedbatch operation using *Clostridium acetobutylicum* suspension culture as biocatalyst for enhancing hydrogen production. Biotechnology Progress 19, 383–388.

Chu, C.F., Li, Y.Y., Xu, K.Q., Ebie, Y., and Inamori, Y. (2008). A pH-and temperature-phased two-stage process for hydrogen and methane production from food waste. International Journal of Hydrogen Energy 33, 4739-4746.

Classen, P.M.A., van Groenestijn, J.W., Janssen, A.J.H., Niel, E.W.J., and Wijffels, R.H. (2000). Feasibility of biological hydrogen production from biomass for utilization in fuel cells. Proceedings of the 1st World Conference and Exhibition on Biomass for Energy and Industry. Sevilla, Spain.

Das, D. (2009). Advances in biohydrogen production processes: An approach towards commercialization. International Journal of Hydrogen Energy 34, 7349-7357.

Das, D., and Veziroglu, T.N. (2001). Hydrogen production by biological processes: a survey of literature. International Journal of Hydrogen Energy 26, 13-28.

Davila-Vazquez, G., Alatriste-Mondragón, F., de León-Rodríguez, A., and Razo-Flores, E. (2008). Fermentative hydrogen production in batch experiments using lactose, cheese whey and glucose: influence of initial substrate concentration and pH. International Journal of Hydrogen Energy 33, 4989-4997.

De Gioannis, G., Muntoni, A., Polettini, A., and Pomi, R. (2013). A review of dark fermentative hydrogen production from biodegradable municipal waste fractions. Waste Management 33, 1345-1361.

de Jager, F.S.J. (1978). Coal Reserves of the Republic of South Africa, Geological Survey. Bulletin 74, 289-330.

de Vrije, T.T., and Claasen, P.M.A. (2003). Dark hydrogen fermentation, Biomethane and bio-hydrogen. Dutch Biological Hydrogen Foundation. The Hague, Netherlands.

Department of Environmental Affairs, (2014). National waste information. Baseline Report. Pretoria, South Africa.

Department of Health, (2014). Available at: http://www.health.gov.za/. (Accessed 18 March 2016).

Devesa-Rey, R., Vecino, X., Varela-Alende, J.L., Barral, M.T., Cruz, J.M., and Moldes, A.M. (2011). Valorization of winery waste vs. the costs of not recycling. Waste Management Research 31, 2327-2335.

Dong, L., Zhenhong, Y., Yongming, S., Xiaoying, K., and Yu, Z. (2009). Hydrogen production characteristics of the organic fraction of municipal solid wastes by anaerobic mixed culture fermentation. International Journal of Hydrogen Energy 34, 812-820.

Eberhard, A.A. (2011). The future of South African coal: market, investment, and policy challenges, Stanford University: Program and Energy and Sustainable Development. Available at: https://fsi.stanford.edu/sites/default/files/WP100EberhardFutureofSouthAfricanCoal.pdf. (Accessed 18 March 2016).

Eker, S., and Sarp, M. (2016). Hydrogen gas production from waste paper by dark fermentation: Effects of initial substrate and biomass concentrations. International Journal of Hydrogen Energy 42, 2562-2568.

Eklund, B., Anderson, E.P., Walker, B.L., and Burrows, D.B. (1998). Characterization of landfill gas composition at the fresh kills municipal solid-waste landfill. Environmental Science and Technology 32, 2233-2237.

Elbeshbishy, E., Hafez, H., Dhar, B.R., and Nakhla, G. (2011). Single and combined effect of various pretreatment methods for biohydrogen production from food waste. International Journal of Hydrogen Energy 36, 11379-11387.

Elsharnouby, O., Hafez, H., Nakhla, G., and El Naggar, M.H. (2013). A critical literature review on biohydrogen production by pure cultures. Internatioanl Journal of Hydrogen Energy 38, 4945-4966.

Energy Information Administration, (2015). Available at: http://www.eia.gov/. (Accessed 18 March 2016).

Eroglu, E., Gunduz, U., Yucel, M., and Eroglu, I. (2009). Photosynthetic bacterial growth and productivity under continuous illumination or diurnal cycles with olive mill wastewater as feedstock. International Journal of Hydrogen Energy 35, 5293–5300.

Eskom, (2014). Integrated Results. Available at: http://www.eskom.co.za/OurCompany/MediaRoom/Documents/IRpresentation2014.pdf. (Accessed 18 March 2016).

Esteghlalian, A.R., Mansfield, S.D., and Saddler, J.N. (2002). Cellulose: Agents for fiber modification or bioconversation? The effect of substrates accessibility on cellulose enzymatic hydrolyzability. Biotechnology in the Pulp and Paper Industry 21, 21-36.

Faloye, F.D., Gueguim Kana, E.B., and Schmidt, S. (2014). Optimization of biohydrogen inoculum development via a hybrid pH and microwave treatment technique – Semi pilot scale production assessment. International Journal of Hydrogen Energy 39, 56074-5616.

Fang, H.H., Li, C., and Zhang, T. (2006). Acidophilic biohydrogen production from rice slurry. International Journal of Hydrogen Energy 31, 683-692.

Fang, H.H.P., Liu, H., and Zhang, T. (2002). Characterization of a hydrogen-producing granular sludge. Biotechnology and Bioengineering 78, 44–52.

Fluri, T. (2009). The potential of concentrating solar power in South Africa. Energy Policy 37, 5075-5080.

Fourie, C.J.S., du Plessis, S.J., and Henry, G. (2006). New airborne geophysical data from the Waterberg Coalfield –South Africa's major future energy source. Available at: https://researchspace.csir.co.za/dspace/handle/10204/2568. (Accessed 18 March 2016).

Ghimire, A., Frunzo, L., Pontoni, L., d'Antonio, G., Lens, P.N.L., Esposito, G., and Pirozzi, F. (2015). Dark fermentation of complex waste biomass for biohydrogen production by pretreated thermophilic anaerobic digestate. Journal of Environmental Management 152, 43-48.

Ghimire, A., Frunzo, L., Pontoni, L., d'Antonio, G., Lens, P.N.L., and Esposito, G. (2015). Dark fermentation of complex waste biomass for biohydrogen production by pretreated thermophilic anaerobic digestate. Environmental Management 152, 43-48.

Gomez, X., Moran, A., Cuetos, M., and Sanchez, M.E. (2006). The production of hydrogen by dark fermentation of municipal solid wastes and slaughterhouse waste: a two-phase process. Journal of Power Sources 157, 727-732.

Goncalves, L.M.D., Barreto, M.T.O., Xavier, A.M.B.R., Carrondo, M.J.T., and Klein, J. (1992). Inert support for lactic acid fermentation. A technological assessment. Applied Microbiology and Biotechnology 38, 305–311.

Guo, X.M., Trably, E., Latrille, E., Carrere, H., and Steyer, J.P. (2010). Hydrogen production from agricultural waste by dark fermentation: a review. International Journal of Hydrogen Energy 35, 10660-10673.

Hallenbeck, P.C., and Ghosh, D. (2009). Advances in fermentative biohydrogen production: the way forward? Trends in Biotechnology 27:287-297.

Han, S.K., and Shin, H.S. (2004). Biohydrogen production by anaerobic fermentation of food waste. International Journal of Hydrogen Energy 29, 569-577.

Hartnady, C. (2010). South Africa’s Diminishing Coal Reserves. South African Journal of Science 106, 18-22.

Hassan, M.B., and Gueguim Kana, E.B. (2016). Design, implementation and assessment of a novel bioreactor for fermentative biohydrogen process development. International Journal of Hydrogen Energy 41, 10136-10144.

Hawkes, F., Dinsdale, R., Hawkes, D., and Hussy, I. (2002) Sustainable fermentative hydrogen production: challenges for process optimisation. International Journal of Hydrogen Energy 27, 1339-1347.

Hong, C., and Haiyun, W. (2010). Optimization of volatile fatty acid production with co-substrate of food wastes and dewatered excess sludge using response surface methodology. Bioresource Technology 101, 5487-5493.

Hsu, T.A., Ladisch, M.R., and Tsao, G.T. (1980). Alcohol from cellulose. ChemTech Journal 10, 315–319.

Hung, C.H., Lee, K.S., Cheng, L.H., Huang, Y.H., Lin, P.J., and Chang, J.S. (2007). Quantitative analysis of a high-rate hydrogen-producing microbial community in anaerobic agitated granular sludge bed bioreactors using glucose as substrate. Applied Microbiology and Biotechnology 75, 693-701.

Hussy, F.R., Hawkes, R., and Dinsdale, R. (2005).Continuous fermentative hydrogen production from sucrose and sugarbeet. International Journal of Hydrogen Energy 34, 471-483.

Hydrogen South Africa, (2016). Available at: http://www.hysainfrastructure.org/. (Accessed 18 March 2016).

International Energy Agency, (2013). Available at: https://www.iea.org/publications/freepublications/publication/CO2EmissionsfromFuelCombustion_Highlights_2016.pdf. (Accessed 18 March 2016).

International Energy Agency, (2015). Available at: https://www.iea.org/publications/freepublications/publication/KeyWorldStatistics2015.pdf. (Accessed 18 March 2016).

Ismail, F., Abd-Aziz, S., MeiLing, C., and Hassan, M.A. (2009). Statistical optimization of biohydrogen production using food waste under thermophilic conditions. Open Renewable Energy Journal 2, 124-131.

Ivanova, G., Rakhely, G., and Kovacs, K.L. (2009). Thermophilic biohydrogen production from energy plants by *Caldicellulosiruptor saccharolyticus* and comparison with related studies. International Journal of Hydrogen Energy 34, 3659–3670.

Jeffrey, L.S. (2005). Characterization of the coal resources of South Africa. Journal of South African Institute of Minerals and Metallurgy 3, 95-102.

Jimenez, A.M., Borja, R., and Martin, A. (2004). A comparative kinetic evaluation of the anaerobic digestion of untreated molasses and molasses previously fermented with *Penicillium decumbens* in batch reactors. Biochemistry Engineering Journal 18, 121-132.

Kamalaskar, L.B., Dhakephalkar, P.K., Meher, K.K., and Ranade, D.R. (2010). High biohydrogen yielding *Clostridium* sp. DMHC-10 isolated from sludge of distillery waste treatment plant. International Journal of Hydrogen Energy 35, 10639–10644.

Kapdan, I.K., and Kargi, F. (2006). Bio-hydrogen production from waste materials. Enzyme and Microbial Technology 38, 569-582.

Kemfert, C., and Schill, W.P. (2009). An analysis of methane mitigation as a response to climate change. Copenhagen, Denmark.

Khanal, S.K., Chen, W.H., Li, L., and Sung, S. (2004). Biological hydrogen production: effects of pH and intermediate products. International Journal of Hydrogen Energy 29, 1123-1131.

Khanna, N., and Das, D. (2013). Biohydrogen production by dark fermentation. Wiley Interdisciplinary Reviews: Energy and Environment 2, 401-421.

Khanna, N., Kotay, S.M., Gilbert, J.J., and Das, D. (2011). Improvement of biohydrogen production by *Enterobacter cloacae* IIT-BT 08 under regulated pH. Journal of Biotechnology 152, 9-15.

Kim, D.H., Wu, J., Jeong, K.W., Kim, M.S., and Shin, H.S. (2011). Natural inducement of hydrogen from food waste by temperature control. International Journal of Hydrogen Energy 36, 10666-10673.

Kim, J.K., Nhat, L., Chun, Y.N., Chun, Y.N., and Kim, S.W. (2008). Hydrogen production conditions from food waste by dark fermentation with *Clostridium beijerinckii* KCTC 1785. Biotechnology and Bioprocess Engineering 13, 499-504.

Kim, S.H., Han, S.K., and Shin, H.S. (2004). Feasibility of biohydrogen production by anaerobic co-digestion of food waste and sewage sludge. International Journal of Hydrogen Energy 29, 1607-1616.

Kisielewska, M., Wysocka, I., and Rynkiewicz, M.R. (2013). Continuous Biohydrogen and Biomethane Production from Whey Permeate in a Two-Stage Fermentation Process. Environmental Progress and Sustainable Energy 33, 4111-1418.

Kleerebezem, R., and van Loosdrecht, M. (2007). Mixed culture biotechnology for bioenergy production. Current Opinions in Biotechnology 18, 207-212.

Kotsopoulos, T.A., Fotidis, I.A., Tsolakis, N., and Martzopoulos, G.G. (2009). Biohydrogen production from pig slurry in a CSTR reactor system with mixed cultures under hyper-thermophilic temperature (70 °C). Biomass and Bioenergy 33, 1168-1174.

Kumar, G.R., and Chowdhary, N. (2016). Biotechnological and Bioinformatics approaches for augmentation of biohydrogen production: A review. Renewable and Sustainable Energy Reviews 56, 1194-1206.

Kumar, N., and Das, D. (2000). Enhancement of hydrogen production by *Enterobacter cloacae* IIT-BT 08. Process Biochemistry 35, 589-593.

Kumar, N., Ghosh, A., and Das, D. (2001). Redirection of biochemical pathways for the enhancement of H_2 production by *Enterobacter cloacae*. Biotechnology Letters 23, 537–541.

Lalaurette, E., Thammannagowda, S., Mohagheghi, A., Mohagheghi, A., Maness, P.C., and Logan, B.E. (2009). Hydrogen production from cellulose in a two-stage process combining fermentation and electrohydrogenesis. International Journal of Hydrogen Energy 34, 6201-6210.

Lay, C.H., Sen, B., Chen, C.C., Wu, J.H., Lee, S.C., and Lin, C.Y. (2012). Co-fermentation of water hyacinth and beverage wastewater in powder and pellet form for hydrogen production. Bioresource Technology 135, 610-615.

Lay, J.J., Lee, Y.J., and Noike, T. (1999). Feasibility of biological hydrogen production from organic fraction of municipal solid waste. Water Research 33, 2579-2586.

Lee, D.Y., Ebie, Y., and Xu, K.Q. (2010). Continuous H_2 and CH_4 production from high-solid food waste in the two-stage thermophilic fermentation process with the recirculation of digester sludge. Bioresource Technology 101, 42-47.

Lee, K.S., Wu, J.F., Lo, Y.S., Lin, P.J., and Chang, J.S. (2004). Anaerobic hydrogen production with an efficient carrier-induced granular sludge bed bioreactor. Biotechnology and Bioengineering 87, 648–657.

Lewis, A.J., Ren, S., Ye, X., Kim, P., Labbe, N., and Borole, A.P. (2015). Hydrogen production from switchgrass via an integrated pyrolysis-microbial electrolysis process. Bioresource Technology 195, 231-241.

Lin, C.N., Wu, S.Y., and Chang, J.S. (2006). Fermentative hydrogen production with a draft tube fluidized bed reactor containing silicone-gel-immobilized anaerobic sludge. International Journal of Hydrogen Energy 3, 2200–2210.

Lin, C.Y., and Lay, C.H. (2004). Carbon/nitrogen-ratio effect on fermentative hydrogen production by mixed microflora. International Journal of Hydrogen Energy 29, 41–45.

Lin, C.Y., Wu, S.Y., Lin, P.J., Chang, J.S., Hung, C.H., and Lee, K.S. (2011). A pilot-scale high-rate biohydrogen production system with mixed microflora. International Journal of Hydrogen Energy 36, 8758-8764.

Lin, P.Y., Whang, L.M., Wu, Y.R., Ren, W.J., Hsiao, C.J., Li, S.L., and Chang, J.S. (2007). Biological hydrogen production of the genus *Clostridium*: Metabolic study and mathematical model simulation. International Journal of Hydrogen Energy 32, 1728-1735.

Liu, D., Zeng, R.J., and Angelidaki, I. (2008). Effects of pH and hydraulic retention time on hydrogen production versus methanogenesis during anaerobic fermentation of organic household solid waste under extreme-thermophilic temperature (70° C). Biotechnology and Bioengineering 100, 1108-1114.

Liu, H., and Wang, G. (2012). Hydrogen production of a salt tolerant strain *Bacillus* sp. B2 from marine intertidal sludge. World Journal of Microbiology and Biotechnology 28, 31-37.

Liu, M., Yuan, Y., Zhang, L.X., Zhuang, L., Zhou, S.G., and Ni, J.R. (2010). Bioelectricity generation by a Gram-positive *Corynebacterium* sp. strain MFC03 under alkaline condition in microbial fuel cells. Bioresource Technology 101, 1807-1811.

Liu, X., Zhu, Y., and Yang, S.T. (2006). Butyric acid and hydrogen production by *Clostridium tyrobutyricum* ATCC 25755 and mutants. Enzyme and Microbial Technology 38, 521-528.

Lo, Y.C., Lee, K.S., Lin, P.J., and Chang, J.S. (2009). Bioreactors configured with distributors and carriers enhance the performance of continuous dark hydrogen fermentation. Bioresource Technology 100, 4381-4387.

Madigan, M.T., Martinko, J.M., Parker, J., Stahl, D.A., and Clark, D.P. (1997). Biology of microorganisms. Prentice Hall, New Jersey, USA.

Mafuleka, S., and Gueguim Kana, E.B. (2015). Modelling and optimization of xylose and glucose production from Napier grass using hybrid pre-treatment techniques. Biomass Bioenergy 77, 200-208.

Manikkandan, T., Dhanasekar, R., and Thirumavalavan, K. (2009). Microbial production of Hydrogen from sugarcane Bagasse using *Bacillus* sp. International Journal of ChemTech Research 1, 344-348.

Massanet-Nicolau, J., Dinsdale, R., Guwy, A., and Shipley, G. (2015). Utilising biohydrogen to increase methane production, energy yields and process efficiency via two stage anaerobic digestion of grass. Bioresource Technology 189, 379-383.

Masset, J., Calusinska, M., Hamilton, C., Hiligsmann, S., Joris, B., and Thornat, P. (2012). Fermentative hydrogen production from glucose and starch using pure strains and artificial co-cultures of *Clostridium* spp. Biotechnology for Biofuels 5, 1-15.

Meher Kotay, S., and Das, D. (2008). Biohydrogen as a renewable energy resource-prospects and potentials. International Journal of Hydrogen Energy 33, 258-263.

Mohanakrishna, G., Venkata Mohan, S., and Sarma, P.N. (2010). Utilizing acid-rich effluents of fermentative hydrogen production process as substrate for harnessing bioelectricity: An integrative approach. International Journal of Hydrogen Energy 35, 3440 – 3449.

Moodley, P., and Gueguim Kana, E.B. (2015). Optimization of xylose and glucose production from sugarcane leaves (*Saccharum officinarum*) using hybrid pretreatment techniques and assessment for hydrogen generation at semi-pilot scale. International Journal of Hydrogen Energy 40, 3859-3867.

Morimoto, M., Atsuko, M., Atif, A.A.Y., Ngan, M.A., Razi, A.F., Iyuke, S.E., and Bakir, A.M. (2004). Biological production of hydrogen from glucose by natural anaerobic microflora. International Journal of Hydrogen Energy 29, 709-713.

Nandi, R., and Sengupta, S. (1998). Microbial production of hydrogen – an overview. Critical Reviews in Microbiology 24, 61–84.

Nath, K., and Das, D. (2004). Improvement of fermentative hydrogen production: various approaches. Applied Microbiology and Biotechnology 65, 520–529.

Nazlina, H.M.Y., Nor'Aini, A.R. Ismail, F., Yusof, M.Z.M. and Hassan, M.A. (2009). Effect of different temperature, initial pH and substrate composition on biohydrogen production from food waste in batch fermentation. Asian Journal of Biotechnology 1, 42-50.

Netherlands Environmental Assessment Agency, (2015). Available at: http://edgar.jrc.ec.europa.eu/newsdocs/jrc-2015-trends-in-global-CO2-emissions-2015-report-98184.pdf. (Accessed 18 March 2016).

Ngoma, L., Masilela, P., Obazu, F.O., and Gray, V.M. (2011). The effect of temperature and effluent recycle rate on hydrogen production by undefined bacterial granules. Bioresource Technology 102, 8986-8991.

Nouni, M.R. (2012). Hydrogen energy and fuel cell technology: Recent development and future prospects in India. Renewable Energy Akshay Urja 5, 10-14.

Obazu, F.O., Daramola, M.O., and Gray, V. (2015). Biohydrogen Production by Bacterial Granules Adapted to grow at Different Thermophilic Temperatures. Proceedings of the World Congress on Engineering, Volume 2. London, United Kingdom.

Oh, S.E., Van Ginkel, S., and Logan, B.E. (2003). The relative effectiveness of pH control and heat treatment for enhancing biohydrogen gas production. Environmental Science and Technology 37, 5186-5190.

Oh, Y.K., Kim, S.H., Kim, M.S., and Park, S. (2004). Thermophilic biohydrogen production from glucose with trickling biofilter. Biotechnology and Bioengineering 88, 690–698.

Okamoto, M., Miyahara, T., Mizuno, O., and Noike, T. (2000). Biological hydrogen potential of materials characteristic of the organic fraction of municipal solid wastes. Water Science and Technology 41, 25-32.

Ozmihci, S., and Kargi, F. (2010). Bio-hydrogen production by photo-fermentation of dark fermentation effluent with intermittent feeding and effluent removal. International Journal of Hydrogen Energy 35, 6674–6680.

Pan, J., Zhang, R., El-Mashad, H.M., Sun, H., and Ying, Y. (2008). Effect of food to microorganism ratio on biohydrogen production from food waste via anaerobic fermentation. International Journal of Hydrogen Energy 33, 6968-6975.

Pandu, K., and Joseph, S. (2012). Comparisons and limitations of biohydrogen production processes: a review. Research Journal of Biotechnology 2, 342–356.

Park, J.L., Lee, J., Sim, S.J., and Lee, J.H. (2009). Production of Hydrogen from Marine Macro-algae Biomass Using Anaerobic Sewage Sludge Microflora. Biotechnology and Bioprocess Engineering 14, 307-315.

Pattra, S., Sangyoka, S., and Boonmee, M. (2008). Bio-hydrogen production from the fermentation of sugarcane bagasse hydrolysate by *Clostridium butyricum*. International Journal of Hydrogen Energy 33, 5256–5265.

Perego, P., Converti, A., Palazzi, E., Borghi, M.B., and Ferraiolo, M. (1989). Fermentation of hardwood hemicellulose hydrolysate by *Pachysolen tannophilus, Candida shehatae* and *Pichia stipitis.* Bioprocess Engineering 22, 205–213.

Roy, S., Kumar, K., Ghosh, S., and Das, D. (2014). Thermophilic biohydrogen production using pre-treated algal biomass as substrate. Biomass and Bioenergy 61, 157-166.

Sagnak, R., and Kargi, F. (2011). Photo-fermentative hydrogen gas production from dark fermentation effluent of acid hydrolyzed wheat starch with periodic feeding. International Journal of Hydrogen Energy 36, 4348–53.

Sarkar, S., and Kumar, A. (2010). Large-scale biohydrogen production from bio-oil. Bioresource Technology 101, 7350–7361.

Salerno, M.B., Park, W., Zuo, Y., and Logan, B.E. (2006). Inhibition of biohydrogen production by ammonia Water Research 40, 1167–1172.

Sangyoka, S., Reungsang, A., and Moonamart, S. (2007). Repeated-batch fermentative for biohydrogen production from cassava starch manufacturing wastewater. Pakistan Journal of Biological Sciences 10, 1782-1789.

Saratale, G.D., Chen, S.D., Lo, Y.C., Saratale, R.G., and Chang, S.C. (2008). Outlook of biohydrogen production from lignocellulosic feedstock using dark fermentation-a review. Science 345, 1326-1330.

Sekoai, P.T., and Daramola, M.O. (2015). Biohydrogen production as a potential energy fuel in South Africa. Biofuel Research Journal 6, 223-226.

Sekoai, P.T., and Gueguim Kana, E.B. (2013). A two-stage modelling and optimization of biohydrogen production from a mixture of agro-municipal waste. International Journal of Hydrogen Energy 38, 8657-8663.

Sekoai, P.T., and Gueguim Kana, E.B. (2014a). Fermentative biohydrogen modelling and optimization in light of miniaturized parallel bioreactors-Review. Biotechnology and Biotechnological Equipments 27, 3901-3908.

Sekoai, P.T., and Gueguim Kana, E.B. (2014b). Semi-pilot scale production of hydrogen from Organic Fraction of Solid Municipal Waste and electricity generation from process effluents. Biomass and Bioenergy 60, 156-163.

Sekoai, P.T., and Yoro, K.O. (2016). Biofuel development Initiatives in Sub-Saharan Africa: Opportunities and Challenges. Climate 4, 1-13.

Sekoai, P.T., Awosusi, A.A., Yoro, K.O., Singo, M., Oloye, O., Ayeni, A.O., Bodunrin, M., and Daramola, M.O. (2017). Microbial cell immobilization in biohydrogen production–A short overview. Critical Reviews in Biotechnology, 1-15.

Sekoai, P.T., Yoro, K.O., and Daramola, M.O. (2016). Batch fermentative biohydrogen production process using immobilized anaerobic sludge from organic solid waste. Environments 3, 1-10.

Sewsynker, Y., and Gueguim Kana, E.B. (2015). Modelling of biohydrogen generation in microbial electrolysis cells (MECs) using a committee of artificial neural networks (ANNs). Biotechnology and Biotechnological Equipments 29, 1208-1215.

Shah, A.T., Favaro, L., Alibardi, L., Cagnin, L., Sandon, A., and Cossu, R. (2016). *Bacillus* sp. strains to produce bio-hydrogen from the organic fraction of municipal solid waste. Applied Energy 176, 116–124.

Sheridan, C.M., Glasser, D., Hildebrandt, D., Petersen, J., and Rohwer, J. (2010). An Annual and Seasonal Characterisation of Winery Effluent in South Africa. South African Journal of Enology and Viticulture 3, 1-8.

Shin, H.S., and Youn, J.H. (2005). Conversion of food waste into hydrogen by thermophilic acidogenesis. Biodegradation 16, 33-44.

Shin, H.S., Kim, S.H., and Paik, B.C. (2003). Characteristics of hydrogen production from food waste and waste activated sludge. Journal of Water and Environmental Technology 1, 177-187.

Show, K.Y., Lee, D.J., and Chang, J.S. (2011). Bioreactor and process design for biohydrogen production. Bioresource Technology 102, 8524-8533.

Singh, L., and Wahid, Z. (2014). Enhancement of hydrogen production from palm oil mill effluent via cell immobilization technique. International Journal of Energy Research 39, 215-222.

Sinha, P., and Pandey, A. (2011). An evaluative report and challenges for fermentative biohydrogen production. International Journal of Hydrogen Energy 36, 7460–7478.

South African Institute for Advanced Materials Chemistry, (2016). Available at: http://www.uwc.ac.za/Faculties/NS/SAIAMC/Pages/default.aspx. (Accessed 18 March 2016).

Tanisho, S., Suzuki, Y., and Wakao, N. (1987). Fermentative hydrogen evolution by *Enterobacter aerogenes* strain E. 82005. International Journal of Hydrogen Energy 12, 623-627.

Tawfik, A., and El-Qelish, M. (2012). Continuous hydrogen production from co-digestion of municipal food waste and kitchen wastewater in mesophilic anaerobic baffled reactor. Bioresource Technology 114, 270-274.

Temudo, M.F., Kleerebezem, R., and van Loosdrecht, M. (2007). Influence of the pH on (open) mixed culture fermentation of glucose: A chemostat study. Biotechnology and Bioengineerong 98, 69-79.

Tenca, A., Schievano, A., Perazzolo, F., Adani, F., and Oberti, R. (2011) Biohydrogen from thermophilic co-fermentation of swine manure with fruit and vegetable waste: Maximizing stable production without pH control. Bioresource Technology 102, 8582-8588.

United Nations, (2009). Policies and measures to realize industrial energy efficiency and mitigate climate change. Available at: http://www.unido.org/fileadmin/user_media/Services/Energy_and_Climate_Change/EPU/UN. (Accessed 18 March 2016).

United Nations, (2015). Framework Convention on Climate Change. Available at: http://unfccc.int/meetings/parisnov2015/meeting/8926.php. (Accessed 18 March 2016).

United States Department of Energy, (2015). Available at: https://www.hydrogen.energy.gov/production.html. (Accessed 18 March 2016).

Uyar, B., Gurgan, M., Ozgur, E., Gunduz, U., Yucel, M., and Eroglu, I. (2015). Hydrogen production by hup^{-} mutant and wild-type strains of *Rhodobacter capsulatus* from dark fermentation effluent of sugar beet thick juice in batch and continuous photobioreactors. Bioprocess and Biosystem Engineering 38, 1935–1942.

Van Ginkel, S.W., Oh, S.E., and Logan, B.E. (2005). Biohydrogen gas production from food processing and domestic wastewaters. International Journal of Hydrogen Energy 30, 1535-1542.

Van Ginkel, S.W., Sung, S., and Lay, J.J. (2001). Biohydrogen production as a function of pH and substrate concentration. Environmental Science and Technology 35, 4726-4730.

Vardar-Schara, G., Maeda, T., and Wood, T.K. (2008). Metabolically engineered bacteria for producing hydrogen via fermentation. Microbial Biotechnology 1, 107–125.

Venetsaneas, N., Antonopoulou, G., Stamatelatou, K., Kornaros, M., and Lyberatos, G. (2009). Using cheese whey for hydrogen and methane generation in a two-stage continuous process with alternative pH controlling approaches. Bioresource Technology 100, 3713-3717.

Venkata Mohan, S., Lalit Babu, V., and Sarma, P. (2008). Effect of various pretreatment methods on anaerobic mixed microflora to enhance biohydrogen production utilizing dairy wastewater as substrate. Bioresource Technology 99, 59-67.

Viitez, E., Mosquera, J., and Ghosh, S. (2000). Kinetics of accelerated solid-state fermentation of organic-rich municipal solid waste. Water Science and Technology 41, 231-238.

Wang, A., Ren, N., Shi, Y., and Lee, D.J. (2008). Bioaugmented hydrogen production from microcrystalline cellulose using co-culture-*Clostridium acetobutylicum* and *Ethanoigenens harbinense* B49. International Journal of Hydrogen Energy 33, 912-917.

Wang, A., Sun, D., Cao, G., Wang, H., Ren, N., Wu, W.M., and Logan, B.E. (2011). Integrated hydrogen production process from cellulose by combining dark fermentation, microbial fuel cells, and a microbial electrolysis cell. Bioresource Technology 102, 4137-4143.

Wang, C.C., Chang, C.W., Chu, C.P., Lee, D.J., Chang, B.V., and Liao, C.S. (2003). Producing hydrogen from wastewater sludge by *Clostridium bifermentans*. Journal of Biotechnology 102, 83-92.

Wang, H., Wang, J., Fang, Z., Wang, X., and Bu, H. (2010a). Enhanced bio-hydrogen production by anaerobic fermentation of apple pomace with enzyme hydrolysis. International Journal of Hydrogen Energy 35, 8303-8309.

Wang, J., and Wan, W. (2008). Influence of Ni^{2+} concentration on biohydrogen production. Bioresource Technology 99, 8864-8868.

Wang, Y., Wang, H., Feng, X., Wang, X., and Huang, J. (2010b). Biohydrogen production from cornstalk wastes by anaerobic fermentation with activated sludge. International Journal of Hydrogen Energy 35, 3090–3099.

Wei, H., Jingang, H., Hongting, Z., and Yongfeng, L. (2016). Continuous biohydrogen production from waste bread by anaerobic sludge. Bioresource Technology 212, 1–5.

Winkler, H. (2010). South Africa's renewable energy policy roadmaps. Energy Research Centre, University of Cape Town, South Africa. Available at:

https://open.uct.ac.za/bitstream/item/19473/Edkins_South_Africa_039_s_renewable_2010.pdf?sequence=1. (Accessed 18 March 2016).

Wu, S.Y., Hung, C.H., Lin, C.N., Chen, H.W., Lee, A.S., and Chang, J.S. (2006). Fermentative hydrogen production and bacterial community structure in high-rate anaerobic bioreactors containing silicone-immobilized and self-flocculated sludge. Biotechnology and Bioengineering 93, 934-946.

Yang, H., Guo, L., and Liu, F. (2010). Enhanced bio-hydrogen production from corncob by a two–step process: Dark-and photo-fermentation. Bioresource Technology 101, 2049–2052.

Yang, H., Shao, P., Lub, T., Shena, J., Wang, D., and Xub, Z. (2006). Continuous biohydrogen production from citric acid wastewater via facultative anaerobic bacteria. International Journal of Hydrogen Energy 31, 1306–1313.

Yasin, H.M.N., Nor' Aini, A.R., Man, H.C., Yusof, M.Z.M., and Hassan, M.L. (2011). Microbial characterization of hydrogen-producing bacteria in fermented food waste at different pH values. International Journal of Hydrogen Energy 36, 9571-9580.

Yin, D., Liu, W., and Zhai, N. (2014). Anaerobic digestion of pig and dairy manure under photo-dark fermentation condition. Bioresource Technology 166, 373–380.

Yokoi, H., Maki, R., and Hirose, J. (2002). Microbial production of hydrogen from starch-manufacturing wastes. Biomass and Bioenergy 22, 389-395.

Yokoi, H., Ohkawara, T., Hirose, J., Hayashi, S., and Takasaki, Y. (1995). Characteristics of hydrogen production by aciduric *Enterobacter aerogenes* strain HO-39. Journal of Fermentation and Bioengineering 80, 571-574.

Yu, H., Zhu, Z., and Hu, W. (2002). Hydrogen production from rice winery wastewater in an upflow anaerobic reactor by using mixed anaerobic cultures. International Journal of Hydrogen Energy 27, 1359-1365.

Zahedi, S., Sales, D., Romero, L., and Solera, R. (2012). Hydrogen production from the organic fraction of municipal solid waste in anaerobic thermophilic acidogenesis: influence of organic loading rate and microbial content of the solid waste. Bioresource Technology 129, 85-91.

Zhang, H., Bruns, M.A., and Logan, B.E. (2006). Biological hydrogen production by *Clostridium acetobutylicum* in an unsaturated flow reactor. Water Research 40, 728-734.

Zhang, Z.P., Show, K.Y., Tay, J.H., Liang, D.T., and Lee, D.J. (2008). Biohydrogen production with anaerobic fluidized bed reactors – a comparison of biofilm-based and granule-based systems. International Journal of Hydrogen Energy 33, 1559–1564.

Zhang, T., Liu, H., and Fang, H.H.P. (2003). Biohydrogen production from starch in wastewater under thermophilic condition. Environmental Management 69, 149-156.

Zheng, G., Kang, Z., Qian, Y., and Wang, L. (2009). Enhanced biohydrogen generation from organic wastewater containing $NH4^{+}$ by phototrophic bacteria *Rhodobacter sphaeroides* AR–3. Frontiers of Environmental Science and Engineering 3, 387-392.

Zhou, P., Elbeshbishy, E., and Nakhla, G. (2013). Optimization of Biological Hydrogen Production for Anaerobic Co-digestion of Food Waste and Wastewater Biosolids. Bioresource Technology 130, 710-718.

Zhu, H., Parker, W., and Basnar, R. (2008). Biohydrogen production by anaerobic codigestion of municipal food waste and sewage sludges. International Journal of Hydrogen Energy 33, 3651–3659.

Zhu, J., Li, Y., Wu, X., Miller, C., Chen, P., and Ruan, R. (2009). Swine manure fermentation for hydrogen production. Bioresource Technology 100, 5472-5477.

Chapter 3 – Dark fermentative biohydrogen production using solid biowaste materials

In this chapter, results of the evaluation of dark fermentative biohydrogen production using South African solid biowaste materials are presented. The feedstocks comprised of solid waste from the agricultural, industrial and municipal sector. They were distinguished based on their biohydrogen production yield and a suitable feedstock was selected for further studies.

3.1 Introduction

With increasing energy demands, utilization of renewable resources for dark fermentative biohydrogen production is a promising approach for clean energy production while at the same time reducing environmental pollution and waste disposal costs (Yasin et al., 2013). In South Africa, solid biowaste materials from the agricultural, municipal, and industrial sector are seen as potential feedstocks for dark fermentative biohydrogen production due to their accessibility, disposal problems, and nutritional composition (Department of Environmental Affairs, 2014).

Biohydrogen production from solid biowaste feedstocks is well documented in literature. For example, Dong et al. (2009) reported an optimum biohydrogen yield of 134 mL H_2/g VS using rice waste. Xiao et al. (2013) achieved a maximum yield of 155.2 mL H_2/g VS using kitchen waste. In another study, Sattar et al. (2016) reported an experimental yield of 60.6 mL H_2/g VS from rice straw. Nonetheless, the choice of biowaste feedstock is crucial in dark fermentation process because it affects the overall process yields and metabolic pathways (e.g. acidogenesis and solventogenesis). It is important to highlight that the biohydrogen production performance is also affected by the operating variables such as temperature, pH, fermentation time, and substrate concentration (Ren et al., 2006). The influence of these on

biohydrogen production is presented in chapter 4. Therefore, this chapter provides information on the results of biohydrogen production using selected South African solid biowaste effluents like bread, sugarcane, pear, mango, potato, cabbage, apple, mealie-pap, brewery waste and corn-cob, respectively. The outcome of this study confirms some results documented in literature on the use of solid biowaste materials for biohydrogen production. In addition, the solid biowaste effluent that gave the maximum biohydrogen yield was utilized in the subsequent studies.

3.2 Materials and methods

3.2.1 Pretreatment of biowaste substrates

The selected solid biowaste materials used in this study were bread, sugarcane, pear, mango, potato, cabbage, apple, mealie-pap, brewery waste and corn-cob. These were collected from various waste disposal sites across the city of Johannesburg, South Africa. They were dried at ambient temperature and then reduced to small particle size (2.00-2.80 mm) using a milling machine (Retsch GmbH, Germany) and stored in sealed glass bottles for further use.

3.2.2 Biohydrogen-producing inoculum

The anaerobic mixed sludge was acquired from the Bushkoppies Wastewater Treatment Plant in Johannesburg, South Africa. The biohydrogen-consuming methanogenic Archaea contained in the sludge were inhibited by heat pretreatment (90 ^{o}C, 30 minutes) as reported in literature (Faloye et al., 2014). The pretreated sludge was supplemented with a synthetic growth medium consisting of (g/L): glucose 10, $CaCl_2.2H_2O$ 0.25, K_2HPO_4 0.75, KH_2PO_4 0.75, $MgSO_4$ 0.40, NaCl 2.0, and $NaHCO_3$ 4.0. It was cultured for three days to further enhance the population of acidogenic biohydrogen-producing bacteria. This served as inoculum for batch fermentation experiments.

3.2.3 Batch biohydrogen fermentation experiments

Series of batch fermentation experiments were conducted using modified 1 L Erlenmeyer flasks. The batch fermenters were inoculated with 50 mL of inoculum and 450 mL of medium which consisted of 30 g/L of biowaste substrate and the synthetic growth medium mentioned in section 3.2.2. The fermentation conditions for initial pH, temperature, agitation speed, and fermentation time were 6.5, 30.3 ^{o}C, 100 rpm, and 90 hours, respectively. These operating conditions were obtained from data reported in literature (Xia et al., 2016). Prior to fermentation, the batch reactors were purged with nitrogen gas for 5 minutes to remove oxygen in the headspace and were immediately sealed with silicone rubber stoppers. The reactors were immersed in a temperature regulated water-bath shaker to maintain the fermentation temperature. In addition, the experiments were conducted in duplicate for accuracy of data and reduction of experimental error.

3.2.4 DNA extraction of biohydrogen-producing bacteria

The fermentation broth of an optimal substrate was taken during peak biohydrogen production. It was transferred into a sterile vial and stored at -4 ^{o}C for further analysis. Genomic DNA was extracted from the medium using a Power Soil DNA extraction Kit (MO Bio Laboratories, Inc., USA), following the protocol provided by the manufacturer.

3.2.5 PCR amplification and 16S rRNA gene sequence analysis

PCR amplification was conducted using a G-STORM thermal cycler (Vacutec, South Africa) in 25 µl reaction volumes containing 0.5 µl of each primer, 5 µl of DNA, 12.5 µl of 2X KAPA2G Robust HotStart ReadyMix (Kapa Biosystems, South Africa), and 6.5 µl of sterilized Millipore water (Whitehead Scientific, South Africa). The following universal primer set was used: 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTGTTACGACTT-3') targeting the universal consensus 16S rDNA

fragment (Lane, 1991). The amplification consisted of a denaturing step at 95 °C for 3 minutes, 35 cycles of 94 °C for 1 minute, annealing at 65 °C for 90 seconds, elongation at 72 °C for 2 minutes, and final extension step at 72 °C for 10 minutes. The PCR products (1500 bp) were analyzed by electrophoresis at 100 V for 30 minutes in 1% (w/v) agarose gel and visualized under UV light after being stained with SYBR Green dye.

The PCR products were sequenced at the Agricultural Research Council (Pretoria, South Africa), using the ABI 3100 Genetic Analyzer (Applied Biosystems, USA). The obtained 16S rRNA sequence was compared with the database sequence available in the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/). The sequences were aligned using Clustal W and a phylogenetic tree was constructed from these aligned sequences by neighbour-joining method using MEGA 7.0 software (Tamura et al., 2011). The phylogenetic tree shown in Figure 3.3 was generated based on maximum likelihood statistical approach of estimation via the Tamura and Nei (1993) method of substitution.

3.2.6 Analytical methods

The fraction of biogas consisting of hydrogen, carbon dioxide, and methane gas was continuously measured at 1 hour intervals using a portable gas analyzer (E Instruments LLC, Langhorne, USA) equipped with hydrogen, carbon dioxide, and methane gas detectors which have a measuring range of 0-100%. A water displacement method was used to determine the total volume of biogas (H_2, CO_2 and CH_4) displaced during biohydrogen production (Sekoai et al., 2016). Volatile fatty acids (VFAs) were measured using a pre-calibrated Gas Chromatograph (Varian 3300 FID GC, USA) which was fitted with a CP Wax 58 (FFAP) Column (25 m x 0.53 mm). The column temperature was set at 50 ^{o}C for 2 minutes and then increased to 190 ^{o}C at the rate of 15 ^{o}C per minute and maintained for 16 minutes. The

temperature of injection port and detector were 250 and 260 ^{o}C, respectively. Helium was used as a carrier gas at flow rate of 50 mL per minute. The total volatile solids (TVS) and chemical oxygen demand (COD) was calculated using the standard methods (APHA, 1998). pH was measured using a pH Meter Basic 20+ (Crison, Spain). Meanwhile, the carbon, hydrogen, nitrogen and sulphur (CHNS/O) elements contained in these solid effluents were measured using a Flash Analyzer (Thermo Scientific Flash 2000 Organic Elemental Analyzer, USA). Oxygen (wt %) was calculated by the difference of C, H, N, S, which was subtracted from 100.

3.3 Results and discussion

3.3.1 Elemental composition of the selected biowaste materials

An elemental (CHNS/O) analysis was conducted on the selected biowaste materials to understand their organic composition as shown in Table 3.1. The chosen feedstocks are highly abundant in South Africa and form a substantial fraction of the country's biowaste materials. Therefore, it is important to know their organic constituents because they will have an impact on biohydrogen-producing reactions and process yields (Kapdan and Kargi, 2006). Furthermore, it has been highlighted in literature that these elements are crucial during dark fermentation process because they are utilized by the biohydrogen-producing enzymes such as [Fe-Fe]- and [Ni-Fe]-hydrogenases (Dong et al., 2009). The presence of these elements in the studied feedstocks confirms their suitability for biohydrogen production as corroborated in related studies (Dong et al., 2009).

Table 3.1: Elemental composition of the selected biohydrogen-producing biowaste materials.

Composition (%)	C	H	N	S	O
Apple	42.58	6.51	0.36	-	50.55
Bread	40.47	5.94	1.81	-	51.78
Brewery	42	6.19	2.01	-	49.8
Cabbage	39.46	5.45	3.42	1.05	50.62
Corn-cob	42.8	5.88	0.49	-	50.83
Mealie-pap	43.8	6.53	2.36	-	47.31
Mango	53.74	8.05	1.03	-	37.18
Pear	41.89	6.45	0.33	-	51.33
Potato	40.45	5.86	0.3	-	53.39
Sugarcane	42.22	6.37	0.3	-	51.11

-: not detected.

3.3.2 Biohydrogen production from biowaste materials

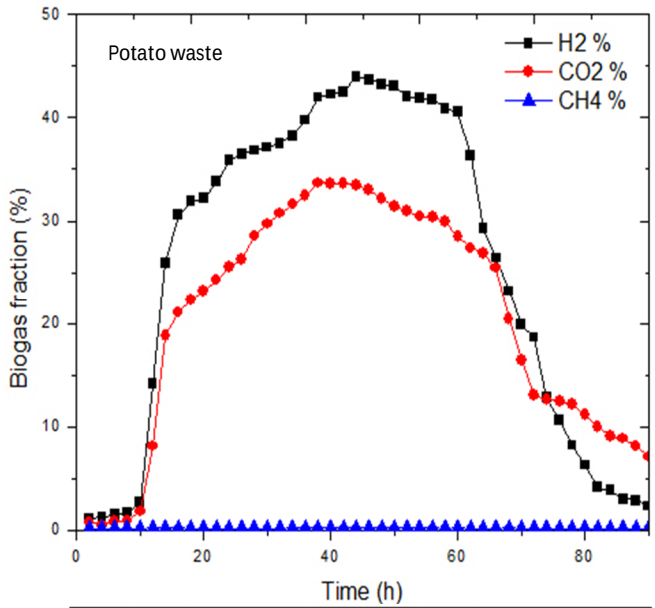
Figure 3.1 shows the fraction of hydrogen, carbon dioxide and methane contained by each feedstock. Amongst the studied substrates; potato, cabbage and brewery waste achieved maximum biohydrogen production. These waste materials are suitable for biohydrogen-producing bacteria because they are rich in carbohydrate content and are easily degradable (Chong et al., 2009). Biohydrogen production started after a short lag phase of 2, 4 and 3 h for potato, cabbage and brewery waste; and reached a peak value of 43.98% at 44 h, 40.32% at 28 h and 38.12% at 26 h, respectively (Figure 3.1). Thereafter, hydrogen production decreased steadily and reached minimum values of 2.35, 2.25 and 4.18%, respectively. This may be due to the accumulation of soluble metabolites such as propionic acid, lactic acid and ethanol which may inhibit biohydrogen-producing reactions (Sekoai and Gueguim Kana, 2014). Methane production was successfully inhibited throughout the process due to thermal pretreatment (90 ^{o}C, 30 minutes) which only enhanced the growth of heat-tolerant spore-forming biohydrogen-producing bacteria (Sekoai and Gueguim Kana, 2013).

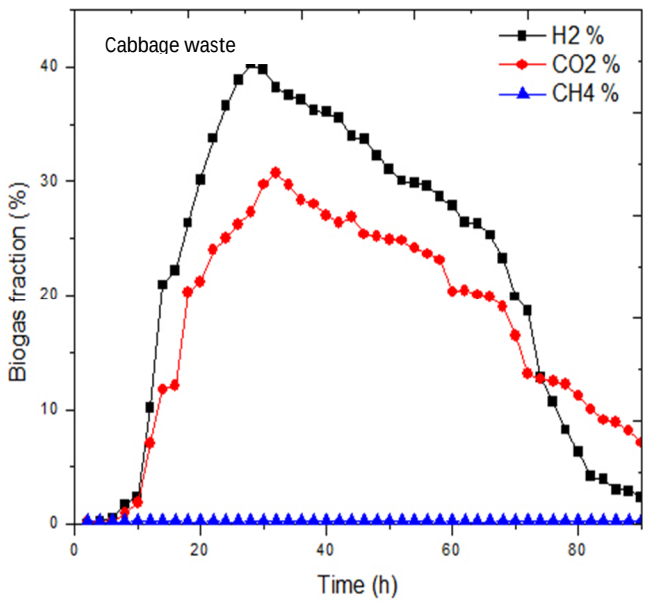
The cumulative biogas for each feedstock increased and reached a plateau as seen in Figure 3.2 as a result of the acidogenic fermentation process. The biohydrogen yield for potato,

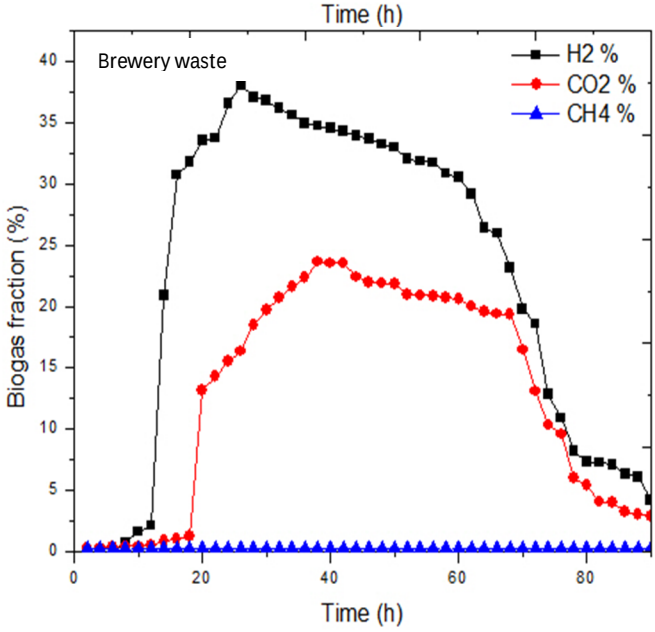
cabbage and brewery waste was 278.36, 238.32 and 215.69 mL H_2/g TVS, respectively. These results coincide with literature. Dong et al. (2009) reported an optimum biohydrogen yields of 134, 106 and 50 mL H_2/g VS using carbohydrate-containing effluents of potato, rice and lettuce waste, respectively, at pH 5.5 and 37 ^oC. The fraction of biohydrogen produced by these effluents was 57–70%, 41–55% and 37–67%, respectively (Dong et al., 2009). Gomez et al. (2006) reported a biohydrogen yield of 52.5–71.3 N L/kg VS using a mixture of organic fraction of municipal solid waste which consisted of 10% banana, 10% apple, 10% orange, 35% cabbage, 25% potatoes, 8% bread and 2% paper. A study by Okamoto et al. (2000) also confirmed a similar biohydrogen production pattern when assessing the potential of rice, carrot, cabbage, chicken skin, eggs, fat and lean meat. An enhanced biohydrogen yield of 26.3, 44.9 and 96 mL H_2/g VS was obtained using carbohydrate-rich substrates of cabbage, carrot and rice, respectively, at pH 7 and 35 ^oC.

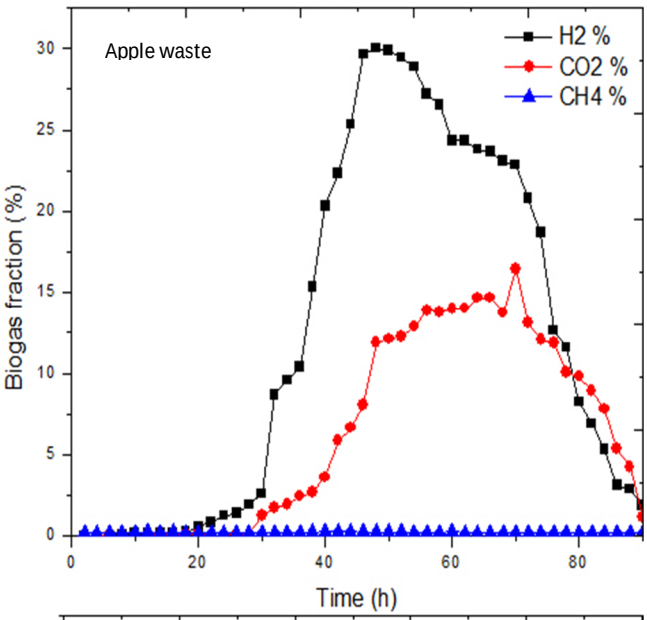
Other studied feedstocks include apple, mango, bread, pear, mealie-pap, corn-cob and sugarcane. The fraction of biohydrogen obtained from these substrates was as follows: apple 30.02%, mango 29.21%, bread 29.05%, pear 29.04%, mealie-pap 28.85%, corn-cob 28.45% and sugarcane 28.32%; and corresponded to a biohydrogen yield of 186.3, 180.26, 175.35, 171.68, 168.74, 156.32 and 153.89 mL H_2/g TVS, respectively. Starch and fruit waste materials are also seen as potential feedstocks for biological hydrogen production due to their high COD content and biodegradable nature (Van Ginkel et al., 2005). Utilization of these substrates is also documented in literature. Feng et al. (2010) reported an optimum biohydrogen yield of 101.08 mL H_2/g TS from apple pomace at pH 7 and 37 ^oC. Akinbomi et al. (2015) evaluated the use of apple, banana, grape and orange waste on biohydrogen production using anaerobic mixed sludge and reported a maximum yield of 504 mL H_2/g VS from apple waste at pH 6.8 and 55 ^oC. In addition, corn-cob and sugarcane waste generated low biohydrogen production yield due to the lignin structure contained in these plant residues

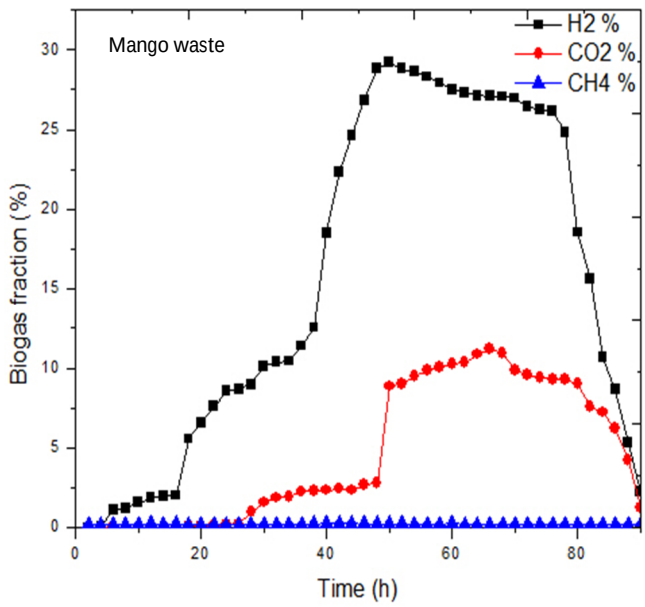
which must be first broken down using various pretreatment methods such as acid, base, heat or ultrasonication to access the fermentable sugars (Das and Veziroglu, 2001; Kapdan and Kargi, 2006). Nevertheless, several authors were able to enhance the production of biohydrogen using pretreated lignocellulose materials (Pawar et al., 2013; Wung et al., 2010; Zhang et al., 2007), although this might impact the process economics of industrial-scale application.

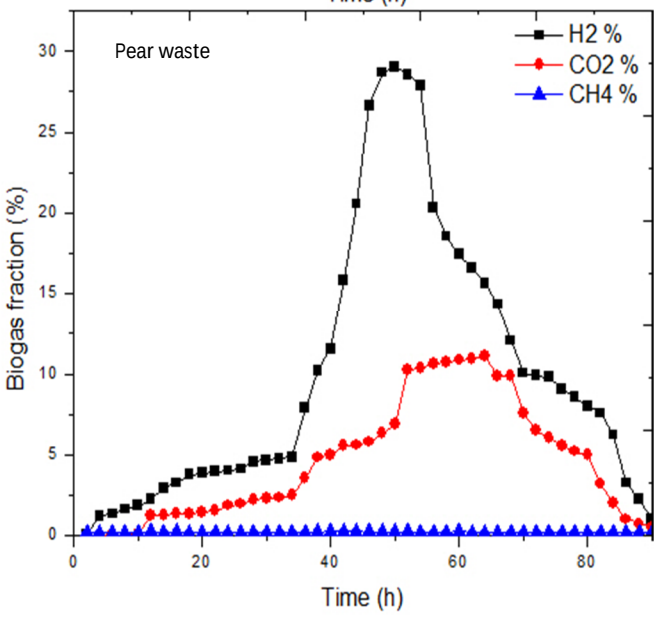

Potato waste
H2 %
CO2 %
CH4 %
Biogas fraction (%)
Time (h)


Cabbage waste
H2 %
CO2 %
CH4 %
Biogas fraction (%)
Time (h)


Brewery waste
H2 %
CO2 %
CH4 %
Biogas fraction (%)
Time (h)


Apple waste
H2 %
CO2 %
CH4 %
Biogas fraction (%)
Time (h)


Mango waste
H2 %
CO2 %
CH4 %
Biogas fraction (%)
Time (h)


Pear waste
H2 %
CO2 %
CH4 %
Biogas fraction (%)
Time (h)

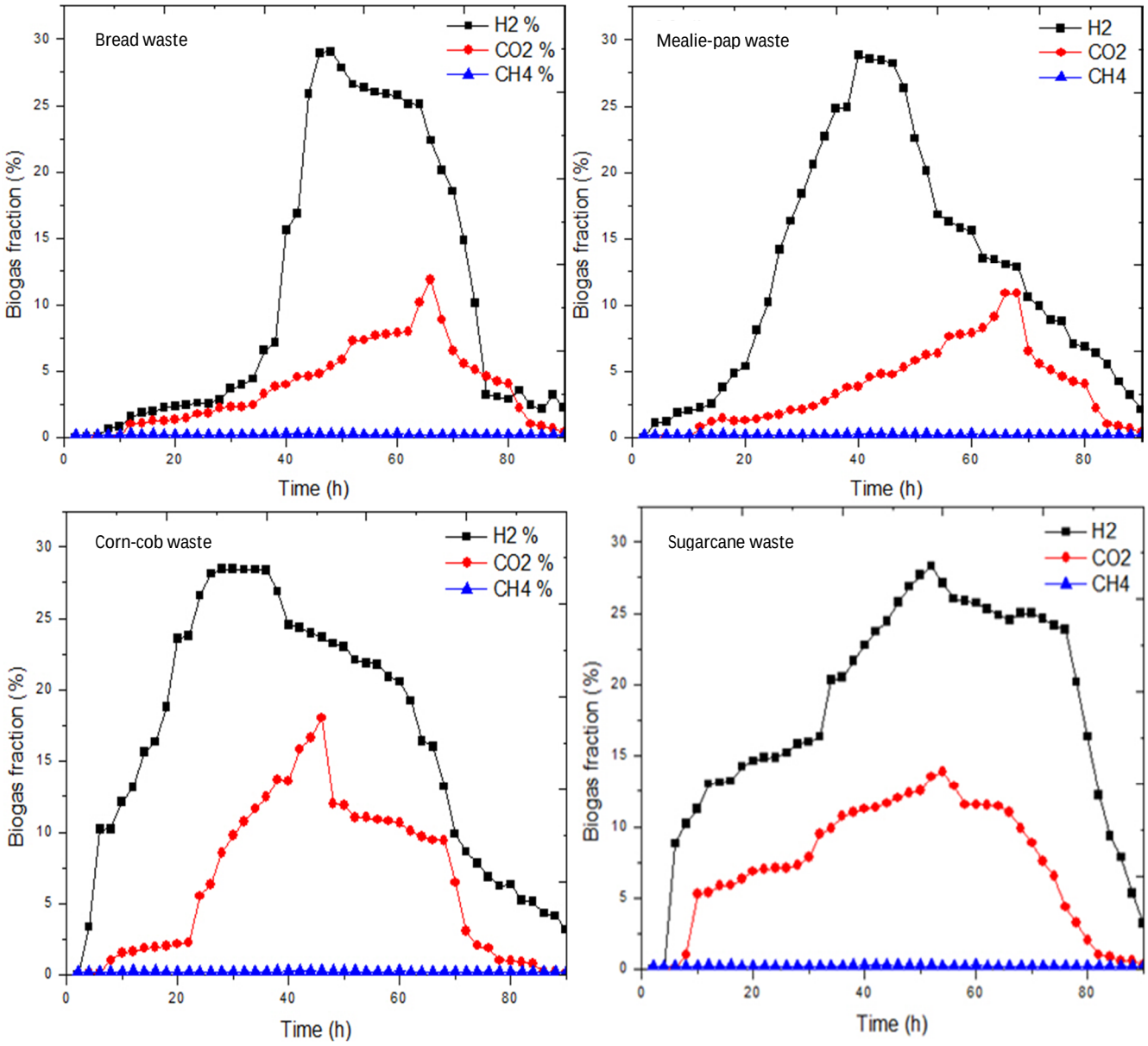
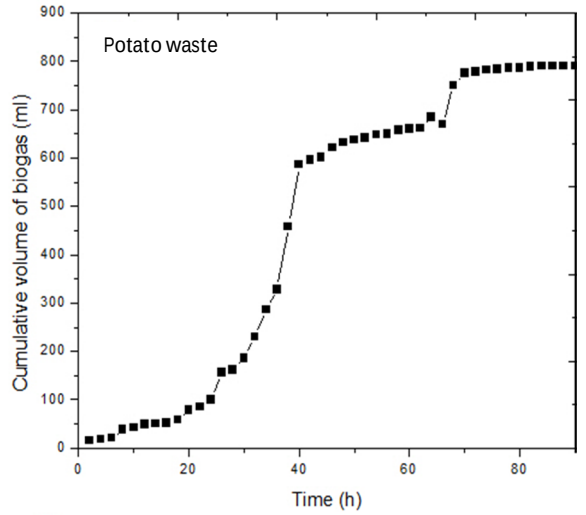
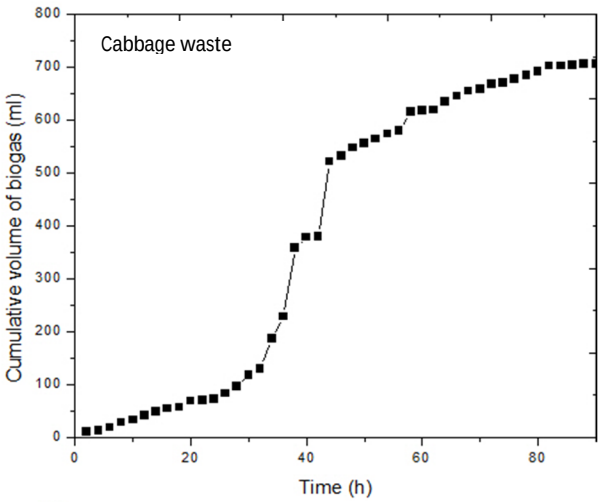
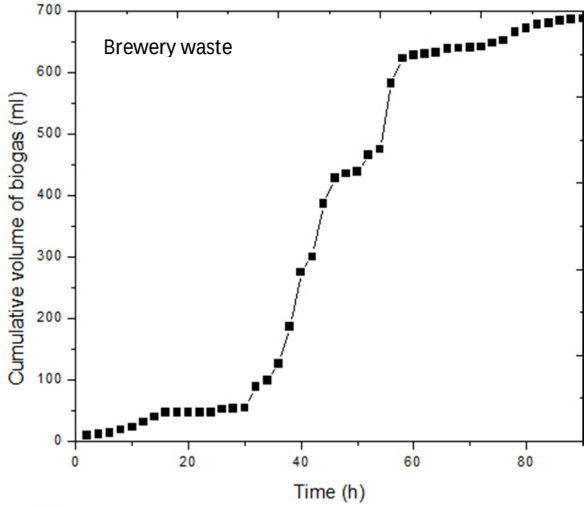
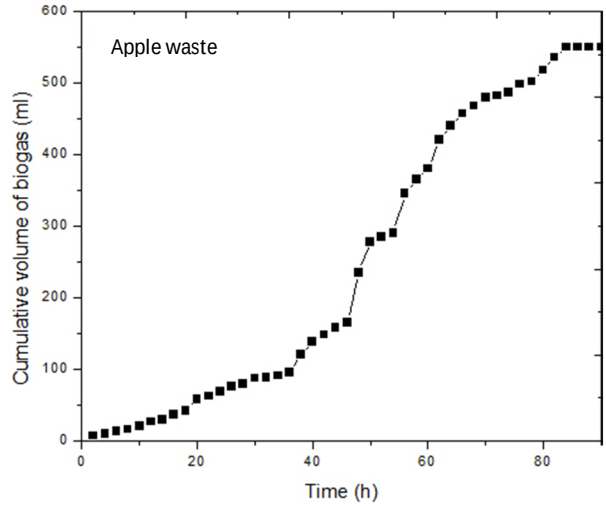


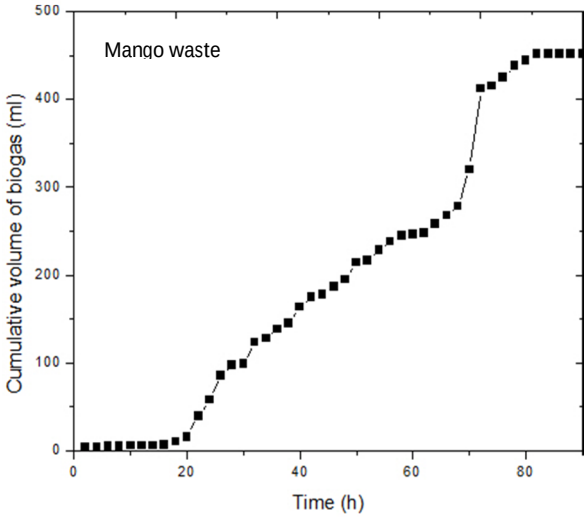
Figure 3.1: Biogas fraction (hydrogen, carbon dioxide and methane) generated from batch fermentation experiments.

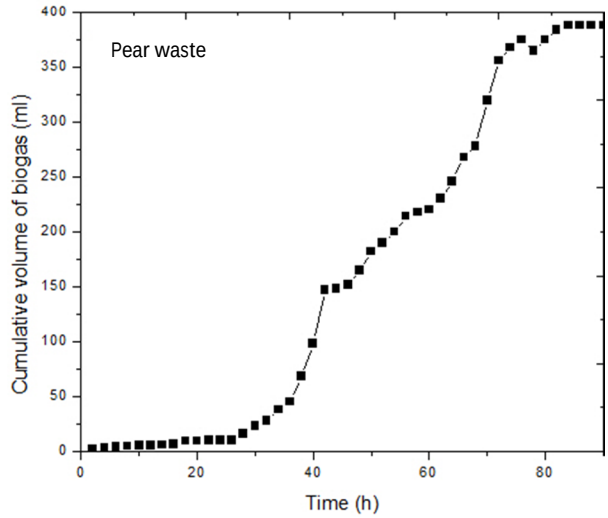

Potato waste
Cumulative volume of biogas (ml)
Time (h)


Cabbage waste
Cumulative volume of biogas (ml)
Time (h)


Brewery waste
Cumulative volume of biogas (ml)
Time (h)


Apple waste
Cumulative volume of biogas (ml)
Time (h)


Mango waste
Cumulative volume of biogas (ml)
Time (h)


Pear waste
Cumulative volume of biogas (ml)
Time (h)

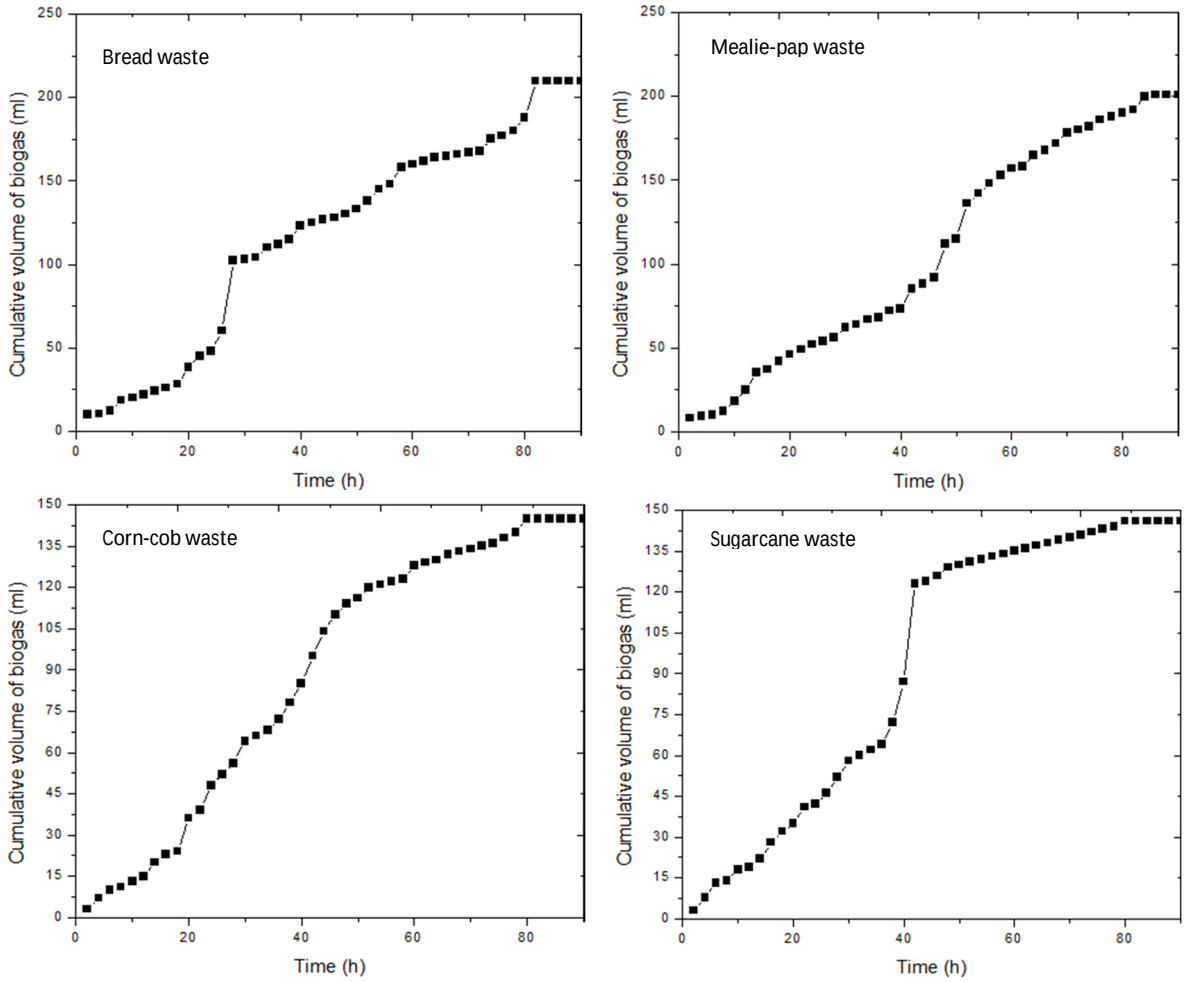


Figure 3.2: Cumulative biogas (hydrogen, carbon dioxide and methane) obtained from batch fermentation experiments.

3.3.3 Volatile fatty acids during batch fermentations

Dark fermentation process is accompanied by volatile fatty acids (VFAs) and alcohol production which reflect changes in metabolic pathways of biohydrogen-producing microorganisms. Thus, process metabolites such as acetate, butyrate, propionate and ethanol accumulate during biohydrogen fermentation process due to acidogenic-solventogenic transition (Sekoai and Gueguim Kana, 2013). These metabolites are used as an indicator for monitoring the biohydrogen production process at lag, exponential, stationary and death phase. The concentrations of VFAs produced during dark fermentative biohydrogen production are shown in Table 3.2. The main VFAs detected were acetate and butyrate which

accounted for 1778.3 and 1253.6 mg/L; 1578.3 and 1224.3 mg/L; 1326.3 and 1092.2 mg/L for potato, cabbage and brewery waste, respectively (Table 3.2). This suggested that acetate and butyrate-type fermentation reactions were adopted by the predominant biohydrogen-producing bacteria, as has been observed for *Clostridium* species (Wu et al., 2006). Hence, these results correlate with literature because 4 mols of hydrogen are produced by the acetate reaction whereas 2 mols of hydrogen are generated from the butyrate reaction as shown in Equations 3.1 and 3.2, respectively. There is high production of biohydrogen in acetate and butyrate fermentation reactions (Feng et al., 2010; Lin et al. 2009).

Acetate: $C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad \Delta G^o = -206.3$ kJ/mol (3.1)

Butyrate: $C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOOH + 2CO_2 + 2H_2 \quad \Delta G^o = -254.8$ kJ/mol (3.2)

A similar observation was reported in related studies. Lin et al. (2009) reported optimum acetate and butyrate concentrations of 97 and 281 mg/L, respectively during peak biohydrogen production from organic fraction of municipal solid waste. Seelert et al. (2015) reported a high acetate-butyrate ratio with low concentration of biohydrogen-inhibiting propionate using food waste. Furthermore, the pH of the fermentation medium was low (3.80-4.82) at the end of each batch process due to a switch in microorganisms from acidogenesis to solventogenesis which is associated with a decrease in pH as shown in Table 3.2 (Chong et al., 2009). The amount of substrate consumed was also evaluated. High substrate consumption was observed in batch experiments using potato, cabbage and brewery waste due to their biodegradable nature as mentioned earlier. A COD removal efficiency of 58.2, 48.6 and 43.2%, respectively, was observed in these experiments (Table 3.2). However, the acidogenic process is still limited by the low substrate conversion (Cheng and Liu, 2011), therefore continuous bioprocesses are usually implemented to prolong biohydrogen production and maximize substrate utilization. Moreso, secondary treatment processes such

as microbial fuel cells and photo-fermentation are incorporated to recover the nutrients contained in the acidogenic effluents (Venkata Mohan, 2009).

Table 3.2: COD removal efficiency, VFAs and final pH at the end of biohydrogen production.

Substrate	COD removal (%)	Acetate (mg/L)	Acetate %	Butyrate (mg/L)	Butyrate %	Propionate (mg/L)	Propionate %	pH
Apple	40.8	1108.5	51.02	985.6	48.56	18.5	0.42	3.80
Bread	37.3	536.8	49.85	475.6	47.67	16.5	2.48	3.98
Brewery	43.2	1326.3	51.87	1092.2	46.32	11.8	1.81	4.11
Cabbage	48.6	1578.3	52.38	1224.3	45.65	32.4	1.97	4.33
Corn-cob	28.6	485.6	45.98	523.6	50.82	16.2	3.2	4.82
Mealie-pap	34.4	511.2	50.08	456.8	48.87	12.2	1.05	4.76
Mango	40.1	987.6	50.38	625.3	48.98	38.2	0.64	4.08
Pear	38.7	892.3	50.12	572.6	49.65	28.9	0.23	4.07
Potato	58.2	1778.3	56.38	1253.6	42.82	36.2	0.8	4.30
Sugarcane	26.7	421.2	44.35	515.3	52.36	14.6	3.29	4.26

3.3.4 Microbial analysis in biohydrogen production using the best substrate

Understanding the presence and functional role of various microorganisms and their association with other taxonomic groups is crucial in biohydrogen production process development. During peak biohydrogen production, the fermentation medium of potato waste (optimal substrate) was analyzed for microbial composition. The phylogenetic analysis showed the dominance of clostridia i.e. the sequence of the isolate (R1S) showed a sequence identity of more than 90% to *Clostridium* species as shown in Table 3.3. These results were also confirmed by the neighbour-joining phylogenetic tree which depicted a close relationship between the isolate and these organisms (Figure 3.3). The presence of members of genus *Clostridium* species found in this study is consistent with literature. These organisms are reported as major biohydrogen-producers during the dark fermentation process (Fang et al., 2002; Maintinguer et al., 2008; Moreno-Davilla et al., 2010; Prasertsan et al., 2009; Venkata Mohan et al., 2011; Wang et al., 2007). Furthermore, they are ubiquitous, versatile, and can thrive at various conditions (Stieglmeier et al., 2009). Members of the genus *Bacillus*

are also prominent in dark fermentation processes and have been identified in many studies. They have been reported to play an important role in hydrolyzing the substrates during the fermentation process which results in high biohydrogen yields (Ueno et al., 2006). Sutthipattanasomboon and Wongthanate (2017) obtained a microbial composition of which more than 47% comprised of isolates sharing > 97% 16 S rRNA sequence identity to *Bacillus cereus*. Zhang et al. (2014) reported that *Bacillus cereus* was the common bacterium during biohydrogen production using starch effluents. It has also been reported that these organisms are capable of depleting the oxygen within the reactor and thus creates suitable anaerobic fermentation conditions for the acidogenic process (Hung et al., 2007). Most biohydrogen production studies documented in literature employ 16S rRNA PCR-DGGE analysis for the identification of bacteria (Babu et al., 2013; Fang et al., 2002; Kim et al., 2006; Wang et al., 2007). This approach is based on the separation of amplified PCR length fragments of specific genes (Hung et al., 2007).

However, the characterization of biohydrogen-producing communities using PCR-based 16S rRNA methods poses some constraints. Firstly, they are biased towards the recovery of certain species i.e. it has been reported in literature that it is difficult to characterize certain Gram positive bacteria (Smith and Osborn, 2009). PCR reaction is governed by melting and renaturation efficiencies of the target DNA sequence and hence the PCR-dependent methods may select certain regions of 16S rRNA as a result of the amplification efficiency (Douterelo et al., 2014). Moreover, slight differences in the primer binding region might cause the bacteria to be undetected (Douterelo et al., 2014). Therefore, PCR-independent methods such as FISH (Leano et al., 2012; O-Thong et al., 2008) and microarrays (Gürgan et al., 2015) are gaining increasing recognition in biohydrogen process development owing to their effectiveness and high throughput data.

Table 3.3: Affiliation of isolate to published species using 16S rRNA sequence.

Organism affiliation	% Query Cover	% Identity	Accession Number
Clostridium sp. LF2	96	97	LK021125.1
Clostridium hydrogeniformans strain DCRUST BT KW-4	94	97	KR997583.1
Clostridiaceae bacterium mt10	96	97	LN846906.1
Clostridium neonatale strain CM-C51	96	94	EU869234.1
Clostridium sp.	96	94	Y15985.1
Clostridium putrefaciens strain JCM 1431	96	94	NR_113324.1
Clostridium neonatale strain CM-C98	96	94	EU869244.1
Desnuesiella massiliensis strain mt10	94	94	NR_144724.1

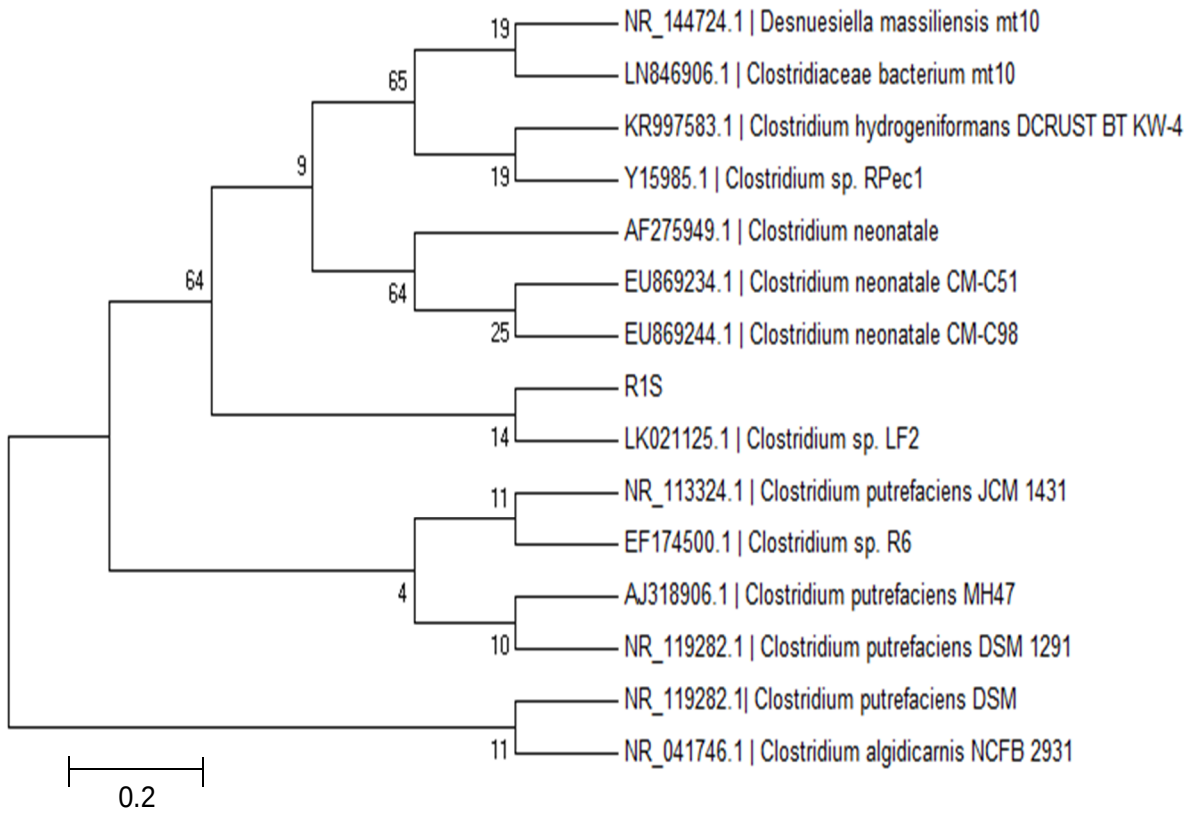


Figure 3.3: Neighbour-joining tree showing the phylogenetic relationship of 16S rRNA sequence of the isolate (R1S) and published sequences of biohydrogen-producing bacteria. The numbers at the branch nodes are bootstrap values (per 1000 trials). The scale bar indicates 0.2 substitutions per site.

3.3.5 Effects of biowaste composition on biohydrogen production

3.3.5.1 Carbohydrate-rich materials

It has been highlighted in various studies that feedstocks rich in carbohydrates are suitable carbon source for biohydrogen-producing bacteria due to their nutritional characteristics which include high moisture content (72-85.2%), high substrate concentration (COD: 19.3 - 346 g/l) and high carbon and nitrogen ratio (9 - 21) (Elbeshbishy et al., 2011; Reungsang and Sreela-or, 2013). They consists of atoms such as carbon, hydrogen and oxygen (see Table 3.1) which play a significant role on active structures of biohydrogen-producing [Fe]-, [Ni-Fe]- and [Fe-Fe]-hydrogenase enzymes during the acidogenic process as indicated earlier (Khanna and Das, 2013). These enzymes enhance the energy metabolism of biohydrogen-producing bacteria and thus enhance the production rate and yield of biohydrogen (Lin et al., 2006). Furthermore, biohydrogen fermentation processes proceed via the anaerobic glycolytic breakdown of sugars such as glucose and sucrose to form acetate and butyrate. Dong et al. (2009) showed that it is thermodynamically favourable to produce biohydrogen from carbohydrate-rich effluents because the Gibbs free energy for these reactions is negative ($\Delta G^{o}<0$) as compared to other feedstocks and thus favours the forward reaction (see Equations 3.1 and 3.2).

3.3.5.2 Lignocellulosic materials

Although lignocellulosic biomass is abundant in nature and is considered an economical feedstock for biohydrogen production, it has to be first pre-treated using various methods (e.g. thermal, acid and alkaline pretreatment) to break down the lignin structure and extract the fermentable sugars such as xylose, glucose and arabinose which are utilized by the biohydrogen-producing microorganisms (Kapdan and Kargi, 2006). This might escalate the process costs at large-scale. Besides, it has been shown in literature that some inhibitors are released during pretreatment and thus affect the biohydrogen production performance (Hsu et

al., 1980); this implies that the type of pretreatment used should be thoroughly assessed.

3.3.5.3 Protein and lipid-rich materials

In contrast to carbohydrate containing substrates, feedstocks rich in lipids and proteins are not suitable for dark fermentation process because they produce reactions that are not thermodynamically favourable (Okamoto et al., 2000). For example, the hydrolysis of lipids produces long chain fatty acids which have an inhibitory effect on biohydrogen-producing bacteria i.e. triacylglycerol is the main component of lipids and consist of 10% glycerol and 90% of long chain fatty acids (Okamoto et al. 2000). It is therefore difficult to produce biohydrogen from these long chain fatty acids because they are not easily metabolized by biohydrogen-producers (Okamoto et al., 2000). Proteins are hydrolyzed to form various amino acids via three types of reactions which are the Stickland, Reductive and Oxidative deamination reactions (de Vladar, 2012). The drawback of using protein rich substrates is that the amino acids are converted to volatile fatty acids and ammonia which compete with biohydrogen-producing pathways (de Vladar, 2012). However, proteins may be advantageous for biohydrogen production because they provide the essential nitrogen source during the biohydrogen fermentation process (Dong et al., 2009).

3.4 Summary

This study has demonstrated the potential of using South African solid biowaste effluents for dark fermentative biohydrogen production. A maximum biohydrogen fraction of 43.98, 40.32 and 38.12% with a corresponding yield of 278.36, 238.32 and 215.69 mL H_2/g TVS, respectively, was obtained using the carbohydrate-rich substrate of potato, cabbage and brewery waste, respectively. Utilization of these feedstocks could significantly contribute towards biohydrogen process development in South Africa because they are easily accessible, inexpensive, and rich in nutritional content. However, more studies should focus on finding inexpensive pretreatment methods, especially for hydrolysis of lignocellulose-containing

materials, to fully access the monomeric sugars and improve the substrate conversion efficiency. Furthermore, the microbial analysis showed the dominance of the members of genus *Clostridium* which are the main microorganisms that play a role during dark fermentative biohydrogen production as indicated in literature. The inoculum pretreatment process was also effective against the growth of biohydrogen-scavenging bacteria such as methanogenic Archaea while enriching only the spore-formers i.e. *Clostridium* species as documented in this study. For dissemination of contributions described in this chapter to the scientific community, a full manuscript has been published in the Proceedings of the *2nd International Conference on Energy, Environment and Climate Change (ICEECC)*, which was held in Mauritius on the 5th -7th July 2017 (see Appendix A).

References

Akinbomi, J., Wikandari, R., and Taherzadeh, M.J. (2015). Enhanced Fermentative Hydrogen and Methane Production from an Inhibitory Fruit-Flavored Medium with Membrane-Encapsulated Cells. Membranes 5, 616-631.

APHA, (1998). Standard Methods for the Examination of Water and Wastewater. 20th Edition, Washington DC, USA.

Babu, M.L., Venkata Subhash, G., Sarma, P.N., and Venkata Mohan, S. (2013). Bio-electroly tic conversion of acidogenic effluents to biohydrogen: An integration strategy for highe r substrate conversion and product recovery. Bioresource Technology 133, 322-331.

Cheng, X.Y., and Liu, C.Z. (2011). Hydrogen production via thermophilic fermentation of cornstalk by *Clostridium thermocellum.* Energy and Fuels 25, 1714-1720.

Chong, M.L., Sabaratnam, V., Shirai, Y., and Hassan, M.A. (2009). Biohydrogen production from biomass and industrial wastes by dark fermentation. International Journal of Hydrogen Energy 34, 3277-3287.

Das, D., and Veziroglu, T.N. (2001). Hydrogen production by biological processes: a survey of literature. International Journal of Hydrogen of Energy 26, 13-28.

de Vladar, H.P. (2012). Amino acid fermentation at the origin of the genetic code. Biology Direct 7, 6.

Department of Environmental Affairs, (2014). Available at: https://www.environment.gov.za/. (Accessed 18 March 2016).

Dong, L., Zhenhong, Y., Yongming, S., Xiaoying, K., and Yu, Z. (2009). Hydrogen production characteristics of organic fraction of municipal solid wastes by anaerobic mixed culture fermentation. International Journal of Hydrogen Energy 34, 812-820.

Douterelo, I., Boxall, J.B., Deines, P., Sekar, R., Fish, K.E., and Biggs, C.A. (2014). Methodo logical approaches for studying the microbial ecology of drinking water distribution sys

tems. Water Research 65, 134-156.

Elbeshbishy, E., Hafez, H., Dhar, B.R., and Nakhla, G. (2011). Single and combined effect of various pretreatment methods for biohydrogen production from food waste. International Journal of Hydrogen Energy 36, 11379-11387.

Faloye, F.D., Gueguim Kana, E.B., and Schmidt, S. (2014). Optimization of biohydrogen inoculum development via a hybrid pH and microwave treatment technique – Semi pilot scale production assessment. International Journal of Hydrogen Energy 39, 56074-5616.

Fang, H.H., Liu, H., and Zhang, T. (2002). Characterization of a hydrogen-producing granula r sludge. Biotechnology and Bioengineering 78, 44-52.

Feng, X., Wang, H., Wang, Y., Wang, X., and Huang, J. (2010). Biohydrogen production from apple pomace by anaerobic fermentation with river sludge. International Journal of Hydrogen Energy 35, 3058-3064.

Gomez, X., Moran, A., Cuetos, M., and Sanchez, M. (2006). The production of hydrogen by dark fermentation of municipal solid wastes and slaughterhouse waste: a two-phase process. Journal of Power Sources 157,727-732.

Gürgan, M., Erkal, N.F., Özgür, E., Gündüz, U., Eroglu, I., and Yücel, M. (2015). Transcriptional Profiling of Hydrogen Production Metabolism of *Rhodobacter capsulatus* under Temperature Stress by Microarray Analysis. International Journal of Molecular Sciences 16, 13781-13797.

Hsu, T.A., Ladisch, M.R., and Tsao, G.T. (1980). Alcohol from cellulose. ChemTech 10, 315-319.

Hung, C.H., Lee, K.S., Cheng, L.H., Huang, Y.H., Lin, P.J., and Chang, J.S. (2007). Quantitative analysis of a high-rate hydrogen-producing microbial community in

anaerobic agitated granular sludge bed bioreactors using glucose as substrate. Applied Microbiology and Biotechnology 75, 693-701.

Kapdan, I.G., and Kargi, F. (2006). Bio-hydrogen production from waste materials. Enzyme and Microbial Technology 38, 569-582.

Khanna, N., and Das, D. (2013). Biohydrogen production by dark fermentation. Wiley Interdisciplinary Reviews: Energy and Environment 2, 401-421.

Kim, S.H., Han, S.K., and Shin, H.S. (2006). Effect of substrate concentration on hydrogen production and 16S rDNA-based analysis of the microbial community in a continuous fermenter. Process Biochemistry 41, 199-207.

Leano, E.P., Anceno, A.J., and Sandhya Babel, S. (2012). Ultrasonic pre-treatment of palm oil mill effluent: Impact on biohydrogen production, bioelectricity generation and underlying microbial communities. International Journal of Hydrogen Energy 37, 12241-12249.

Lin, C.N., Wu, S.Y., and Chang, J.S. (2006). Fermentative hydrogen production with a draft tube fluidized bed reactor containing silicone-gel-immobilized anaerobic sludge. International Journal of Hydrogen Energy 3, 2200–2210.

Lin, C.N., Wu, S.Y., Chang, J.S., and Chang, J.S. (2009). Biohydrogen production in a three-phase fluidized bed bioreactor using sewage sludge immobilized by ethylene-vinyl acetate copolymer. Bioresource Technology 100, 3298-3301.

Maintinguer, S.I., Fernandes, B.S., Duarte, I.C.S., Saavedra, N.K., Adorno, M.A.T., and Varesche, M.B. (2008). Fermentative hydrogen production by microbial consortium. International Journal of Hydrogen Energy 33, 4309-4317.

Moreno-Davila, I.M.M., Rios-Gonzalez, .L.J., Gaona-Lozano, J.G., Garza-Garcia, Y., de la Garza, J.A.R., and Rodriguez-Martinez, J. (2010). Biohydrogen production by

anaerobic bio films from a pre-treated mixed microflora. Research Journal of Applied Sciences 5, 376-382.

Okamoto, M., Miyahara, T., Mizuno, O., and Noike, T. (2000). Biological hydrogen potential of materials characteristic of the organic fraction of municipal solid wastes. Water Science and Technology 41, 25-32.

O-Thong, S., Prasertsan, P., Karakashev, D., and Angelidaki, A. (2008). 16S rRNA-targeted probes for specific detection of *Thermoanaerobacterium* spp., *Thermoanaerobacterium thermosaccharolyticum*, and *Caldicellulosiruptor* spp. by fluorescent in situ hybridization in biohydrogen producing systems. International Journal of Hydrogen Energy 33, 6082-6091.

Pawar, S.S., Nkemka, V.N., Zeidan, A.A., Murto, M., and van Niel, E.W.J. (2013). Biohydrogen production from wheat straw hydrolysate using *Caldicellulosiruptor saccharolyticus* followed by biogas production in a two-step uncoupled process. International Journal of Hydrogen Energy 38, 9121-9130.

Prasertsan, P., O-Thong, S., and Birkeland, N.K. (2009). Optimization and microbial commu nity analysis for production of biohydrogen from palm oil mill effluent by thermophilic fermentative process. International Journal of Hydrogen Energy 34, 7448-7459.

Ren, N., Li, J., Li, B., Wang, Y., and Liu, S. (2006). Biohydrogen production from molasses by anaerobic fermentation with a pilot-scale bioreactor system. International Journal of Hydrogen Energy 31, 2147-2157.

Reungsang, A., and Sreela-or, C. (2013). Bio-Hydrogen Production from Pineapple Waste Extract by Anaerobic Mixed Cultures. Energies 6, 2175-2190.

Sattar, A., Arslan, C., Ji, C., Sattar, S., Mari, I.A., Rashid, H., and Ilyas, F. (2016). Comparing the Bio-Hydrogen Production Potential of Pretreated Rice Straw Co-Digested with Seeded Sludge Using an Anaerobic Bioreactor under Mesophilic Thermophilic

Conditions. Energies 9, 1-14.

Seelert, T., Ghosh, D., and Yargeau, V. (2015). Improving biohydrogen production using *Clostridium beijerinckii* immobilized with magnetite nanoparticles. Applied Microbiology and Biotechnology 99, 4107–4116.

Sekoai, P.T., and Gueguim Kana, E.B. (2013). A two-stage modelling and optimization of biohydrogen production from a mixture of agro-municipal waste. International Journal of Hydrogen Energy 38, 8657-8663.

Sekoai, P.T., and Gueguim Kana, E.B. (2014). Semi-pilot scale production of hydrogen from Organic Fraction of Solid Municipal Waste and electricity generation from process effluents. Biomass and Bioenergy 60, 156-163.

Sekoai, P.T., Yoro, K.O., and Daramola, M.O. (2016). Batch Fermentative Biohydrogen Production Process Using Immobilized Anaerobic Sludge from Organic Solid Waste. Environments 3, 1-10.

Smith, C.J., and Osborn, M. (2009). Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. FEMS Microbial Ecology, 67, 6-20.

Stieglmeier, M., Wirth, R., Kminek, G., and Moissl-Eichinger, C. (2009). Cultivation of Anaerobic and Facultatively Anaerobic Bacteria from Spacecraft-Associated Clean Rooms. Applied and Environmental Microbiology 75, 3484–3491.

Sutthipattanasomboon, C., and Wongthanate, J. (2017). Enhancement of biohydrogen production from starch processing wastewater and further inside its ecosystem disclosed by 16S rDNA sequencing and FISH. Brazilian Archives of Biology and Technology 60, 1-12.

Tamura, K., and Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution 10, 512-526.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA7: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution 28, 2731-2739.

Ueno, Y., Sasaki, D., Fukui, H., Haruta, S., Ishii, M., and Igarashi, Y. (2006). Changes in bacterial community during fermentative hydrogen and acid production from organic waste by thermophilic anaerobic microflora. Journal of Applied Microbiology 101, 331-343.

Van Ginkel, S.W., Oh, S.E., and Logan, B.E. (2005). Biohydrogen gas production from food processing and domestic wastewaters. International Journal of Hydrogen Energy 30, 1535-1542.

Venkata Mohan, S. (2009). Harnessing of biohydrogen from wastewater treatment using mixed fermentative consortia: Process evaluation towards optimization. International Journal of Hydrogen Energy 34, 7460-7474.

Venkata Mohan, S., Agarwal, L., Mohanakrishna, G., Srikanth, S., Kapley, A., and Purohit, H.J. (2011). Firmicutes with iron dependent hydrogenase drive hydrogen production in anaerobic bioreactor using distillery wastewater. International Journal of Hydrogen Ene rgy 36, 8234-8242.

Wang, X., Hoefel, D., Saint, C.P., Monis, P.T., and Jin, B. (2007). The isolation and microbia l community analysis of hydrogen producing bacteria from activated sludge. Journal of Applied Microbiology 103, 1415–1423.

Wu, S.Y., Hung, C.H., Lin, C.N., Chen, H.W., Lee, A.S., and Chang J.S. (2006). Fermentative hydrogen production and bacterial community structure in high-rate anaerobic bioreactors containing silicone-immobilized and self-flocculated sludge. Biotechnology and Bioengineering 93, 934-946.

Wung, H., Fang, M., Fang, Z., and Bu, H. (2010). Effects of sludge pretreatments and organic acids on hydrogen production by anaerobic fermentation. Bioresource Technology 101, 8731-8735.

Xia, A., Jacob, A., Herrmann, C., and Murphy, J.D. (2016). Fermentative bio-hydrogen production from galactose. Energy 96, 346-354.

Xiao, L., Deng, Z., Fung, K.Y., and Ng, K.M. (2013). Biohydrogen generation from anaerobic digestion of food waste. International Journal of Hydrogen Energy 38, 13907-13913.

Yasin, N.H.M., Mumtaz, T., Hassan, M.A., and Rahman, N.A. (2013). Food waste and food p rocessing waste for biohydrogen production: A review. Journal of Environmental Mana gement 130, 375-385.

Zhang, M.L., Fan, Y.T., Xing, Y., Pan, C.M., Zhang, G.S., and Lay, J.J. (2007). Enhanced biohydrogen production from cornstalk wastes with acidification pretreatment by mixed anaerobic cultures. Biomass Bioenergy 31, 250-254.

Zhang, T., Bao, M., Wang, Y., Su, H., and Tan, T. (2014). Genome Sequence of *Bacillus cereus* Strain A1, an Efficient Starch Utilizing Producer of Hydrogen. Genome Announcements 29, 1-2.

Chapter 4 – Parametric optimization of biohydrogen production from potato waste and scale-up study using immobilized anaerobic mixed bacteria

In this chapter, results of the parametric optimization of biohydrogen production using potato waste via response surface methodology (RSM) approach (using a two-level-four-factor (2^4) central composite design (CCD)) and a scale-up study (using immobilized anaerobic mixed sludge as the inoculum) are presented.

4.1 Introduction

Optimization of operating conditions during biohydrogen production is importance in biohydrogen process development because it helps in enhancing its yields (Sekoai, 2016). Operating variables such as substrate concentration, pH, temperature, and fermentation time have been highlighted as the main variables that influence the overall performance of dark fermentation processes (Pan et al., 2008). The substrate concentration affects the biohydrogen-producing bacteria, the formation of soluble intermediates, and medium pH (Sekoai et al., 2016). The pH affects the substrate conversion efficiency, microbial composition, and hydrogenase activity (Antonopoulou et al., 2008). The temperature of the process affects the substrate hydrolysis, inhibition of biohydrogen-consuming microorganisms, and prevention of volatile fatty acids (Mullai et al., 2013). Meanwhile, the fermentation time controls the activity of acidogenic biohydrogen-producing bacteria (Kim et al., 2006). Thus, these variables should be monitored to minimize the accumulation of biohydrogen-inhibiting reactions (Bundhoo and Mohee, 2016).

Employing statistical techniques such as the response surface methodology approach (RSM) could help in understanding the main and interaction effects of the abovementioned variables, thereby paving the way for the optimization of the process toward achieving a higher

biohydrogen yield. RSM is a statistical tool that assesses the relationship between input variables on response outputs (Moodley and Gueguim Kana, 2017). It has been used in understanding the parametric effect on various bioprocesses such as the production of citric acid from waste (Urak et al., 2015), fermentable sugar production from wood waste (Ayeni et al., 2013), biomethane production (Saleh et al., 2012), yoghurt production (Yaakob et al., 2012), and biohydrogen production (Faloye et al., 2013) to mention but a few and results obtained from the studies have paved the way for the optimization of these processes.

With regards to scale-up studies and design of biological processes, immobilization of inoculum to minimize contamination, maintain anaerobic conditions, and enhance productivity has received much attention over the past few years (Wu and Lin, 2004). Unlike suspended bacterial cells, it offers several process advantages such as high metabolic activity, enhancement in cell density, easy handling, re-usability of cells, enhanced operational stability, enhanced separation of the fermentation broth, and improvement in the overall yields (Leino et al., 2012; Lin et al., 2006; Martins et al., 2013; Wu et al., 2005). Amongst the immobilization matrices, calcium alginate is highly recommended because it is inexpensive, easily available, and offers good biocompatibility (Duarte et al., 2013). However, it has some drawbacks such as weak mechanical stability and large pore size, but these shortcomings can be eliminated by the incorporation of metals, carbon sources, and polymers (Sekoai et al., 2016). In this chapter, the results of optimization and scale-up studies are reported. The parametric effects of potato waste concentration, pH, temperature, and fermentation time on biohydrogen production were investigated via response surface methodology (RSM) approach using central composite design (CCD). Consequently, the operating conditions were optimized. Having established the optimal conditions, an attempt was made to scale-up the biohydrogen production using immobilization technology with a calcium alginate matrix as the support inoculum for biohydrogen production.

4.2 Materials and methods

4.2.1 Experimental design

Potato waste was chosen as a suitable feedstock for biohydrogen production as documented in chapter 3. It was prepared using the method outlined in section 3.2.1. The anaerobic mixed sludge was heat pretreated as described in section 3.2.2. Twenty-six batch experimental runs based on the 2^4-central composite design (CCD) obtained via the use of an experimental software (STATISTICA 8 release 7 statistical software, Statsoft Inc., USA) were conducted and the biohydrogen yields from these runs were calculated. The process variables considered in the design of the experiments were potato waste concentration (10–40 g/L), pH (3–8), temperature (32–38 ^{o}C), and fermentation time (5–120 hours). These values were obtained from the preliminary investigations carried out in our laboratory prior to the extensive study on parametric effect reported in this chapter.

4.2.2 Inoculum preparation and fermentation experiments

The pretreated sludge was supported with a synthetic growth medium consisting of (in g/L): sucrose 10, KCl 0.25, NH_4Cl 0.5, K_2HPO_4 0.5, K_2HPO_4 0.5, $NaHCO_3$ 8.0, $MgSO_4$ 0.312, $CaCl_2.6H_2O$ 0.01. The resulting medium was then transferred into a sterilized 1 L Schott bottle and cultured for 24 hours at 30 ^{o}C using a water-bath shaker to enhance the population of biohydrogen-producing bacteria. The medium was used as inoculum for all the subsequent experiments. The twenty-six batch experimental runs, generated from the 2^4-CCD, were conducted using modified 250 mL Erlenmeyer flask reactors. Substrates were weighed and transferred into sterilized reactors along with 10 mL inoculum and 90 mL distilled water. The experimental conditions were maintained as specified in Table 4.1. The reactors were purged with nitrogen gas for 5 minutes and immediately covered with silicone rubber stoppers to create anaerobic conditions that favour the growth of biohydrogen-producing bacteria (Hallenbeck and Ghosh, 2009). The fermentation temperature was maintained by immersing

the reactors in a temperature-regulated water-bath shaker at 100 rpm. The experiments were conducted in duplicate for accuracy of data and reduction of experimental error.

4.2.3 Determination of biohydrogen production

The biogas fraction consisting of hydrogen, carbon dioxide, and methane was continuously monitored at 1 minute intervals using BCP-H_2, BCP-CO_2, and BCP-CH_4 sensors (Bluesens GmbH, Germany) with a measuring range of 0-100%. The volume of biogas was measured with a milligas counter (Bluesens, Germany). The pH was measured using a pH meter (pH Meter Basic 20+, Crison, Spain). The cumulative biohydrogen volume was determined according to Equation 4.1 (Chong et al., 2009):

$$V_{H,i}= V_{H,i-1} + C_{H,i}(V_{G,i} - V_{G,i-1}) + V_H (C_{H,i} - C_{H,i-1}) \quad (4.1)$$

Where $V_{H,i}$ and $V_{H,i-1}$ represent the cumulative biohydrogen gas volumes at current (i) and previous time interval (i-1), respectively. $C_{H,i}$ and $C_{H,i-1}$ are the fractions of biohydrogen at current (i) and previous (i-1) time interval. $V_{G,i}$ and $V_{G,i-1}$ are the total biogas volumes at current (i) and previous (i-1) time interval, and V_H represents the total volume of headspace in the reactor.

4.2.4 Model development and optimization of operating variables

The obtained experimental results (see Table 4.1), which consist of input variables and their respective biohydrogen yields, were used to generate a second-order polynomial regression model that describes the effects of the variables on the yield. The general second-order polynomial presented in Equation 4.2 was considered in the development of the data-based model to describe the effect of the operating variables on the biohydrogen yield.

$$Y = \alpha_0 + \alpha_1 A + \alpha_2 B + \alpha_3 C + \alpha_4 D + \alpha_{11} A^2 + \alpha_{22} B^2 + \alpha_{33} C^2 + \alpha_{44} D^2 + \alpha_{12} AB + \alpha_{13} AC + \alpha_{14} AD + \alpha_{23} BC + \alpha_{24} BD + \alpha_{34} CD \quad (4.2)$$

Where Y represents the biohydrogen production yield in mL H_2/g TVS; A, the potato waste concentration in g/L; B, the fermentation time in hours; C, the pH; and D, the operating temperature in degree Celsius. α_0 to α_{34} are the regression coefficients; A, B, C and D are the linear terms; A^2 , B^2, C^2 and D^2 are the quadratic terms; and AB, AC, AD, BC, BD and CD are the terms indicating the interactive effect. The regression coefficients of the model were estimated using the Least Squares (LS) estimation technique embedded in the STATISTICA 8 release 7 Statistical Software (Stat Ease Inc., USA). In addition, one-way analysis of variance (ANOVA) was conducted on the developed model to determine its statistical significance. The optimum operating conditions for biohydrogen production were obtained by solving the polynomial regression model using a suitable method as described by Myers and Montgomery (1995). Additional experiments were conducted using the optimum conditions which served as solutions to the developed model, and the results were compared to those of the non-optimum conditions.

4.2.5 Scale-up study

A biohydrogen scale-up study was evaluated using the established optimum operating conditions obtained in section 4.2.4. During the study, the inoculum (sludge) was immobilized on a porous matrix (alginate) to enhance the biohydrogen yield, minimize contamination, and provide good mixing within the reactor (Wu et al., 2005).

4.2.5.1 Immobilization of bacteria

One litre of pretreated sludge was centrifuged at 10 000 rpm for 15 minutes to extract the biohydrogen-producing bacterial cells. The cells were mixed with alginate solution, containing 50 g of alginate powder in 2.5 L of sterilized distilled water along with the harvested cells. The solution was stirred for 2 hours to achieve homogeneity. Alginate solution containing biohydrogen-producing cells was extruded drop-wise through a peristaltic

pump into 1M $CaCl_2$ solution to form oval-like beads (with diameter 6–8 mm). The beads (total weight 830 g) were stored in 1M $CaCl_2$ solution at ambient temperature for further use.

4.2.5.2 Biohydrogen production

Biohydrogen production was conducted using a 13 L Benchtop Labfors INFORS HT Bioreactor (Basel, Switzerland) at a working volume of 10 L using the optimized operating conditions obtained from section 4.2.4. Prior to the fermentation process, the reactor was sterilized by autoclaving at 121 ^{o}C for 15 minutes. The sterilized reactor was fed with 9 L of potato waste medium and 830 g of the immobilized beads. Anaerobic conditions were achieved by flushing the reactor with nitrogen gas for 5 minutes. The initial pH was adjusted (without further control) using 1M NaOH and homogeneity of the mixture within the reactor was achieved by agitating the reactor at 100 rpm continuously. Temperature was maintained by an electric heating jacket that was wrapped around the glass reactor vessel. The biogas produced (hydrogen, carbon dioxide, methane) was analysed as described in section 4.2.3. The schematic representation of biohydrogen production is shown in Figure 4.1.

4.2.5.3 Determination of volatile fatty acids

Liquid broth samples were taken at 1 hour interval from the reactor and analyzed for volatile fatty acids (VFA) using a pre-calibrated Gas Chromatograph (Varian 3300 FID GC, USA) equipped with a CP Wax 58 (FFAP) Column (25 m x 0.53 mm). The initial column temperature was 50 ^{o}C for 2 minutes and increased to 190 ^{o}C at the rate of 15 ^{o}C per minute and maintained for another 16 minutes. The injection temperature and the detector temperature were 250 ^{o}C and 260 ^{o}C, respectively. Helium gas was used as the carrier gas at a flow rate of 50 mL per minute.

4.2.5.4 Morphology of alginate beads before and after fermentation

The used and unused calcium alginate beads were subjected to stereoscopic imaging to understand their morphological changes before and after the fermentation experiments. The images of the samples were captured using a Nikon SMZ745T (Tokyo, Japan) stereomicroscope equipped with NIS-Element D Z-Series 7 Software. The camera was a Nikon DS-Fi2 CCD operated by a Nikon Digital Sight System. Furthermore, the calcium alginate beads were rinsed with distilled water; air dried for 1 hour and the morphological changes due to the fermentation process were investigated using a scanning electron microscope (SEM) (FEI Quanta 200 SEM, USA). The SEM was operated under vacuum between 3.9 $x10^{-4}$ to 2.2 $x10^{-3}$ Pascals with a voltage of 30 kV.

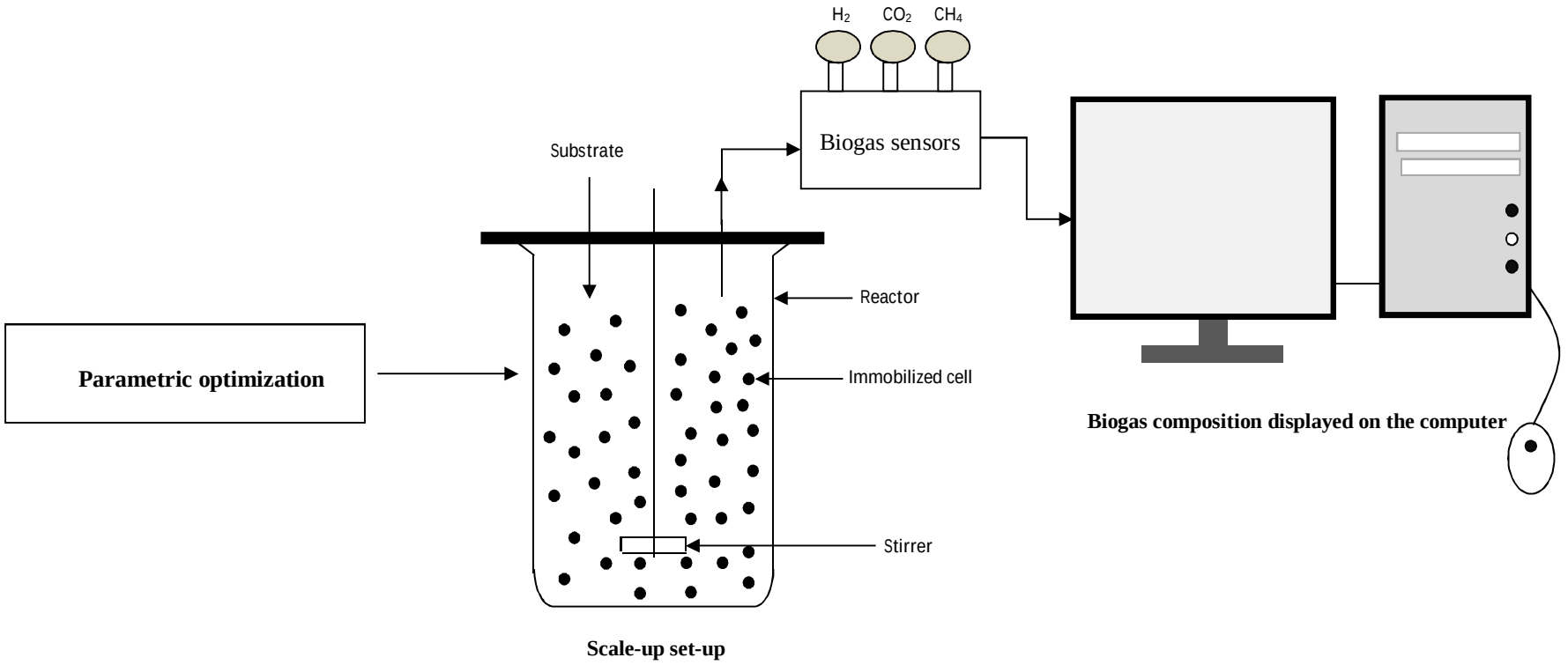


Figure 4.1: Schematic representation of biohydrogen production process.

4.3 Results and discussion

4.3.1 Modelling and effects of operating variables

Experimental data (Table 4.1) obtained from the 2^4-CCD model were used to generate a second-order polynomial regression equation (Equation 4.3) relating the potato waste concentration, pH, temperature and fermentation time to biohydrogen production yield. The statistical significance of the model was also assessed using ANOVA (Table 4.2). A coefficient of determination (R^2) of 0.9924 was obtained, implying that 99.24% of the variability observed in the data can be explained by the model. The statistical significance of the model was further confirmed by the *F* and *P* values of 6.8516 and 0.0212, respectively. The high R^2 value indicates that the model is suitable to explain the production of biohydrogen within the experimental conditions considered in this study (Myers and Montgomery, 1995). The adjusted R^2 value (0.9855) also confirms the validity of the results. Furthermore, Figure 4.2 shows the good agreement of the experimental yield with the model-predicted yield, thereby confirming the validity of the model to describe the behavior observed during the biohydrogen production.

$$Y = 23983.42 + 121.58A - 0.89B - 329.17C - 1406.39D - 0.65A^2 + 30.45C^2 + 21.3D^2 + 0.07AB - 0.32AC - 2.59AD - 0.24BC \quad (4.3)$$

Table 4.1: Experimental design matrix, observed and predicted values of biohydrogen production.

Run	Potato conc. (g/L)	Time (h)	pH	Temp (^{o}C)	Observed H_2 (mL H_2/g TVS)	Predicted H_2 (mL H_2/g TVS)
1	25	5	5	32	55.10	55.10
2	40	62	3	35	48.98	48.98
3	10	62	3	35	42.42	42.42
4	25	62	5	35	39.49	39.49
5	40	120	5	35	27.29	27.29
6	10	62	5	32	17.77	17.77
7	40	120	5.5	35	68.04	68.04
8	40	62	8	35	35.03	35.03
9	40	62	5	32	51.59	51.59
10	25	5	8	35	60.45	60.45
11	25	120	8	35	47.52	47.52
12	40	120	8	38	63.12	63.12
13	25	5	5.5	35	16.88	16.88
14	25	5	8	36	31.27	31.27
15	25	120	8	32	23.25	23.25
16	25	120	3	36	30.06	30.06
17	10	5	5.5	35	26.75	26.75
18	25	5	3	38	14.18	14.18
19	25	62	5.5	38	14.52	14.52
20	40	62.5	5.5	38	24.84	24.84
21	25	120	5	35	54.52	54.52
22	40	62.5	8	35	22.29	22.29
23	10	62.5	5	32	13.40	13.40
24	25	5	3	35	47.71	47.71
25	25	120	5	35	34.78	34.78
26	10	62	8	38	37.64	37.64

Table 4.2: Analysis of variance (ANOVA) for biohydrogen production.

Factor	Sum of squares	Degree of freedom	Mean squares	F-value	P-value
A	574.4	1	574.4	2.6922	0.1248
B	1464.7	1	1464.7	6.8651	0.0212
C	10221.5	1	10221.5	47.9105	0.0000
D	6393.4	1	6393.4	29.9674	0.0001
AB	6162.9	1	6162.9	28.8870	0.0001
AC	407.0	1	407.0	1.9077	0.1905
AD	25816.6	1	25816.6	121.0082	0.0000
BC	4884.1	1	4884.1	22.8928	0.0004
A^2	49555.2	1	49555.2	232.2760	0.0000
B^2	68.9	1	68.9	0.3230	0.5795
C^2	102985.2	1	102985.2	482.7143	0.0000
D^2	29886.2	1	29886.2	140.0832	0.0000
Error	2773.5	13	213.3		
Total SS	368232.4	25			

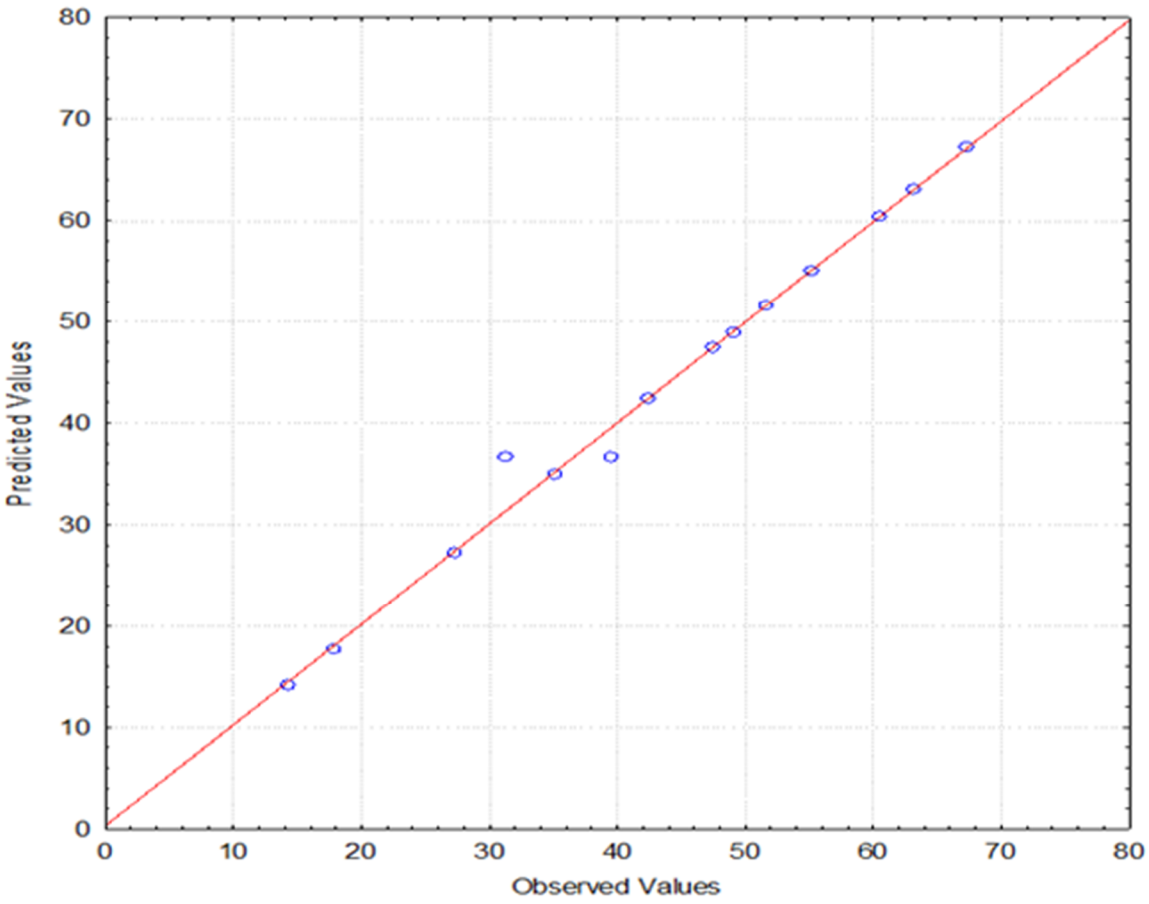


Figure 4.2: Observed (experimental) versus predicted values of biohydrogen production.

4.3.1.1 Main effects of operating variables on biohydrogen production yield

The results showing the main and the linear interactive effect of the studied variables on biohydrogen production are presented in Table 4.1, and are shown on the response surface curves (Figure 4.3 (A-F)). The production of biohydrogen varied from 13.40 to 68.04 mL H_2/g TVS. A high biohydrogen production was achieved in runs 12 and 7 with concomitant yields of 63.12 and 68.04 mL H_2/g TVS, respectively. The main effect of the individual variable on biohydrogen production yield was evaluated as well. It was observed that low fermentation time (5 and 62.5 h), low potato waste concentration (10 and 25 g/L), and low pH were not suitable for biohydrogen production as observed in runs 13, 18, 19, and 23. This could likely be attributed to low pH which has been shown to have an inhibitory effect on the activity of biohydrogen-producing bacteria, and thus lowers the biohydrogen yield (Boboescu et al., 2016; Keskin et al., 2012; Wong et al., 2014). pH has been highlighted as one of the most important parameters in biohydrogen production because it controls various factors such as substrate conversion, activity of the hydrogenase enzymes, buffering capacity of the medium, and the activity of the metabolites (Show et al., 2012).

Moreover, the fermentation time for the experimental runs 13, 18, 19, and 23 was less than 120 h, which implies that the bacteria did not acquire sufficient time to metabolize the substrate. These results are in agreement with literature. Faloye et al. (2013) reported a high biohydrogen yield of 1.89 mol H_2/mol glucose; from potato waste at pH 9.45, whereas low pH between 3 and 4 proved to be unfavourable for biohydrogen production. In the same vein, a study by Rorke and Gueguim Kana (2016) showed a maximum biohydrogen yield of 213.14 mL H_2/g fermentable sugar from sorghum waste at pH 7. It should be noted that protons (H^+) are required during biohydrogen production to maintain optimum levels of adenosine triphosphate (ATP) in bacteria. Therefore, pH should be operated at an optimum condition that will result in maximum uptake of nutrients, proton gradient, and polarity

during its production (Rorke and Gueguim Kana, 2016). Moreover, appropriate pH is required to prevent the growth of bacteria that compete with biohydrogen-producing microorganisms (Dong et al., 2009; Pan et al., 2008). The fermentation time of 120 h was suitable for biohydrogen production as shown in experimental results of runs 7 and 12. These results were also consistent with previous studies (Khanna and Das, 2013). Hence, the above findings highlight the effect of the operating variables on biohydrogen production yields.

4.3.1.2 Interactive effect of operating variables on biohydrogen production yield

The interactive effect of the operating variables (potato waste concentration, fermentation time, pH, and temperature) on biohydrogen production yield, as shown with the contours, is depicted in Figure 4.3 (A–F). These contours show the interaction between two operating variables at a time and the two other variables were fixed at median values (coded value = 0).

For the interactive effect of fermentation time and potato waste concentration on biohydrogen production, it was observed that an increase in both fermentation time (0 to 140 h) and potato waste concentration (5 to 40 g/L) maximized the biohydrogen yield (Figure 4.3 (A)). These results coincide with those of Fan et al. (2004). In the study, the authors achieved a maximum biohydrogen yield at high substrate concentration (20 g/L). Similar observation on biohydrogen improvement at high substrate concentration (12.5 g/L) has been reported by Qiu et al. (2016). High substrate concentrations are usually preferred in biohydrogen process because they enhance the population of spore-forming bacteria during the exponential growth phase (Mafuleka and Gueguim Kana, 2015). However, it has been shown in some studies that high concentrations might inhibits the biohydrogen-producing pathways due to the increased formation of other fermentation by-products (e.g. volatile fatty acids, alcohols) that compete with biohydrogen-producing reactions (Dhillon et al., 2011; Moodley and Gueguim Kana,

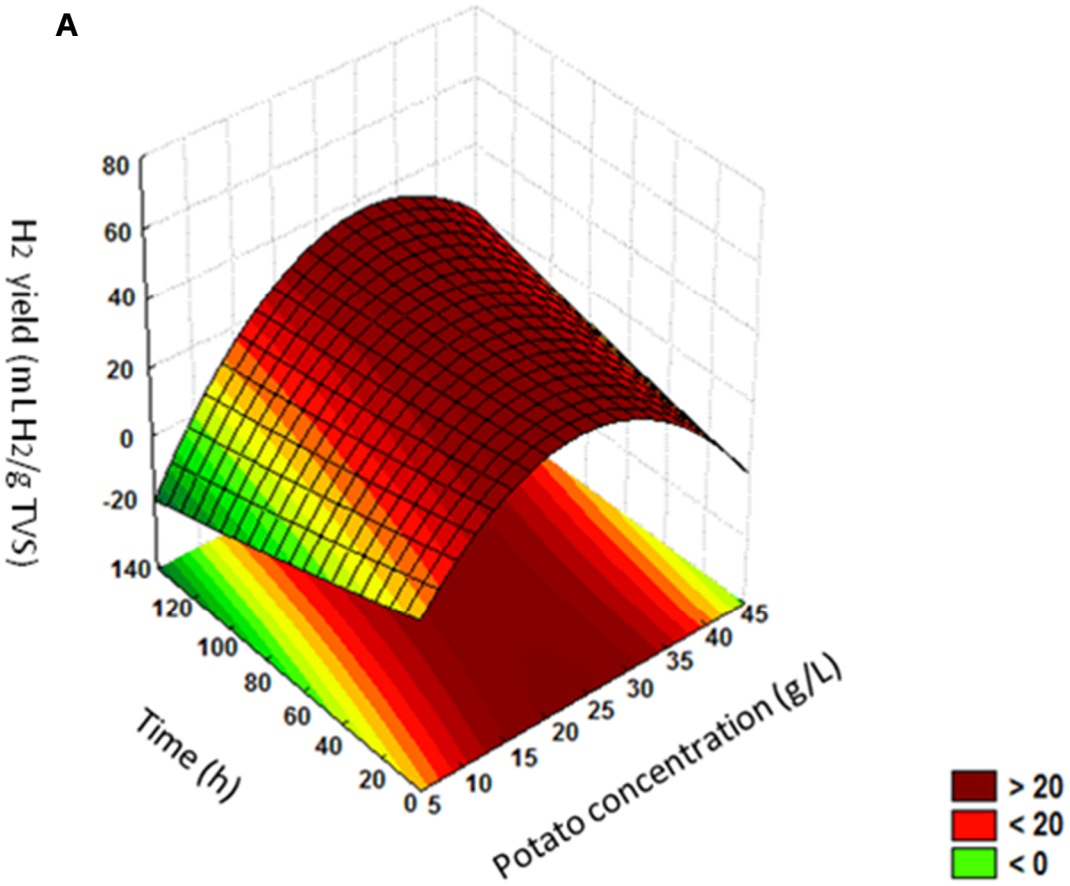
2015; Wu and Lin, 2004). From these reports, it can be deduced that appropriate concentration of substrate is therefore necessary for enhanced biohydrogen production yields.

The contour plot in Figure 4.3 (B) shows the interaction between pH and potato waste concentration on biohydrogen yield. An increase in both pH (between 8 and 9) and potato waste concentration (5–45 g/L) improved the biohydrogen yield. In Figure 4.3 (C), it can be seen that high pH values ranging from 8 to 9 shortened the lag phase, and therefore increased the biohydrogen production. This observation is consistent with previous studies where it has been shown that alkaline medium inhibits the growth of biohydrogen-consuming hydrogenotrophic methanogens, resulting in an enhanced biohydrogen yield (Sekoai and Gueguim Kana, 2014; Sinha and Pandey, 2011). Although fermentation time of 80 to 140 h enhanced the biohydrogen yield (Figure 4.3 (C)), it is crucial to operate at optimum range because a rapid transition from acidogenesis (biohydrogen-producing reactions) to solventogenesis (biohydrogen-inhibiting reactions) during the process could occur (Argun et al., 2008) and terminate hydrogen production.

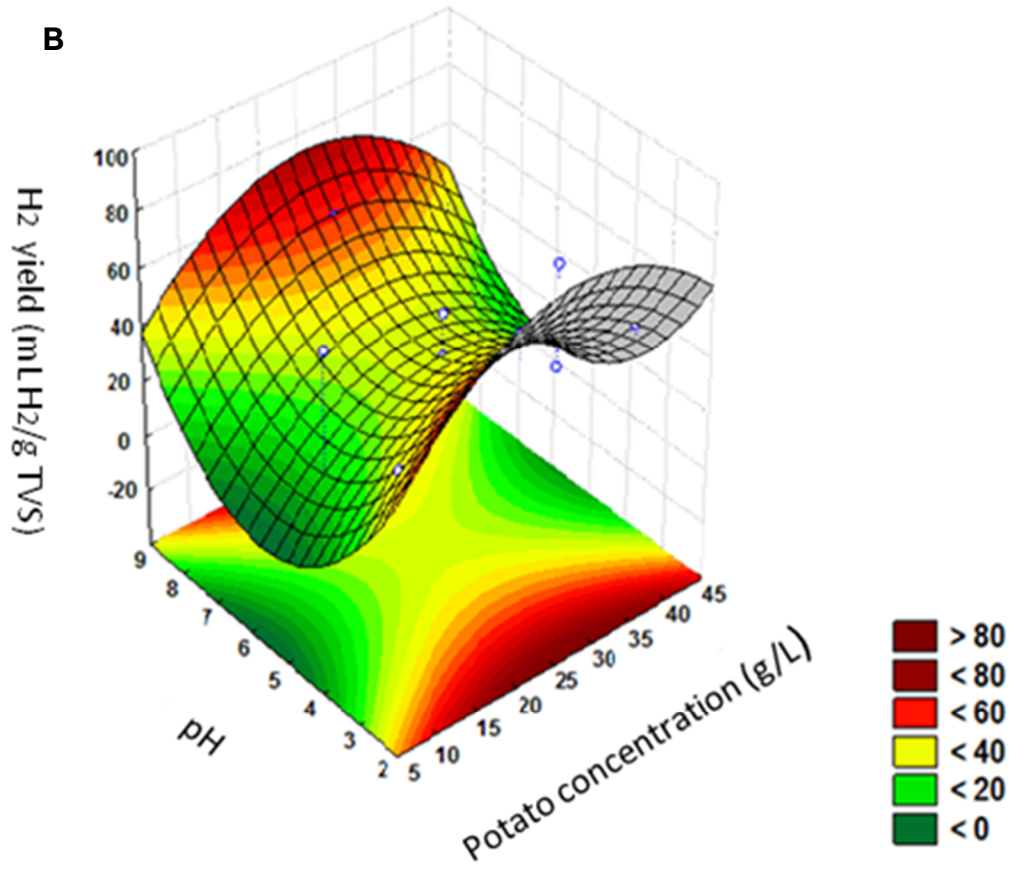
The interaction effect of temperature and fermentation time on biohydrogen yield is depicted in Figure 4.3 (D). It can be seen that high biohydrogen yield (> 80 mL H_2/g TVS) is attainable at temperature between 38–39 °C and fermentation time between 0−140 h. The same observation has been reported by Wang and Wan (2008), where the authors observed an increase in biohydrogen production yield when the temperature was raised from 20 to 35 ^{o}C. However, increasing the temperature beyond 35 ^{o}C (35−55 ^{o}C) resulted in drastic decline in biohydrogen yield; which is attributed to a reduction of the population of mesophilic biohydrogen-producers at high temperatures (Wang and Wan, 2008). Temperature plays a crucial role in biohydrogen production because it controls several factors such as substrate hydrolysis, inhibition of biohydrogen-consuming bacteria i.e. methanogens, homoacetogens,

sulphate-reducing bacteria, and decreases the production of organic acids (Fan et al., 2004; Khanna and Das, 2013; Pan et al., 2008; Qiu et al., 2016). Thermophiles are beneficial in biohydrogen production due to enhanced biohydrogen conversion efficiency and the ability to suppress the biohydrogen-inhibiting reactions (Wang and Wan, 2008). However, they are energy-intensive and will therefore increase the operating costs at large-scale production. On the contrary, mesophiles are highly preferred in biohydrogen production due to their minimum energy requirements. Therefore, more than 60% of biohydrogen production studies are carried out at mesophilic conditions (Elbeshbishy et al., 2011).

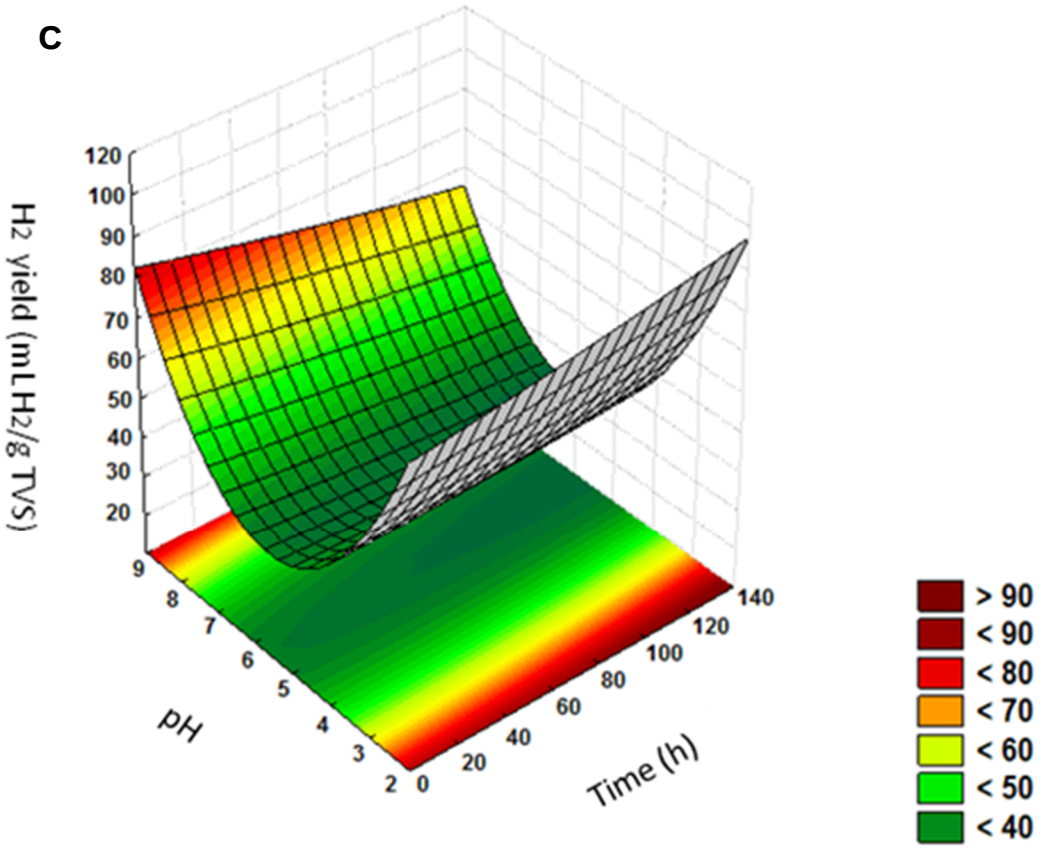
The synergistic effect of temperature and pH on biohydrogen production is shown in Figure 4.3 (E). It can be seen that maintaining high temperature (37–39 °C) and high pH (8–9) maximize the biohydrogen production yield. Furthermore, a high biohydrogen yield of 80 mL H_2/g TVS was obtained at 38–39 °C and 20–45 g/L as observed in Figure 4.3 (F). Therefore, these results demonstrate the importance of understanding the effect of operating variables on biohydrogen yield during dark fermentation process because the information obtained could pave the way for optimization and scale-up study of biohydrogen production from biowaste materials.


A
H2 yield (mL H2/g TVS)
80
60
40
20
0
-20
140
120
100
80
60
40
20
0
Time (h)
5
10
15
20
25
30
35
40
45
Potato concentration (g/L)
> 20
< 20
< 0

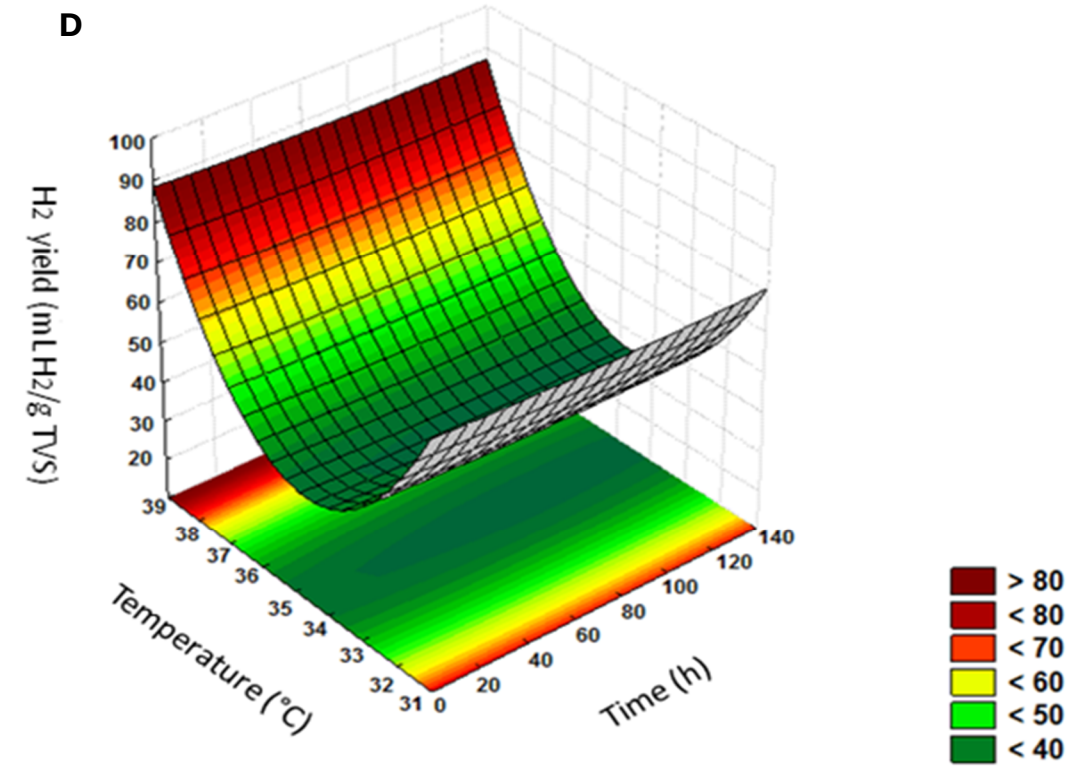
B


H2 yield (mL H2/g TVS)
100
80
60
40
20
0
-20
pH
9
8
7
6
5
4
3
2
Potato concentration (g/L)
5
10
15
20
25
30
35
40
45
> 80
< 80
< 60
< 40
< 20
< 0

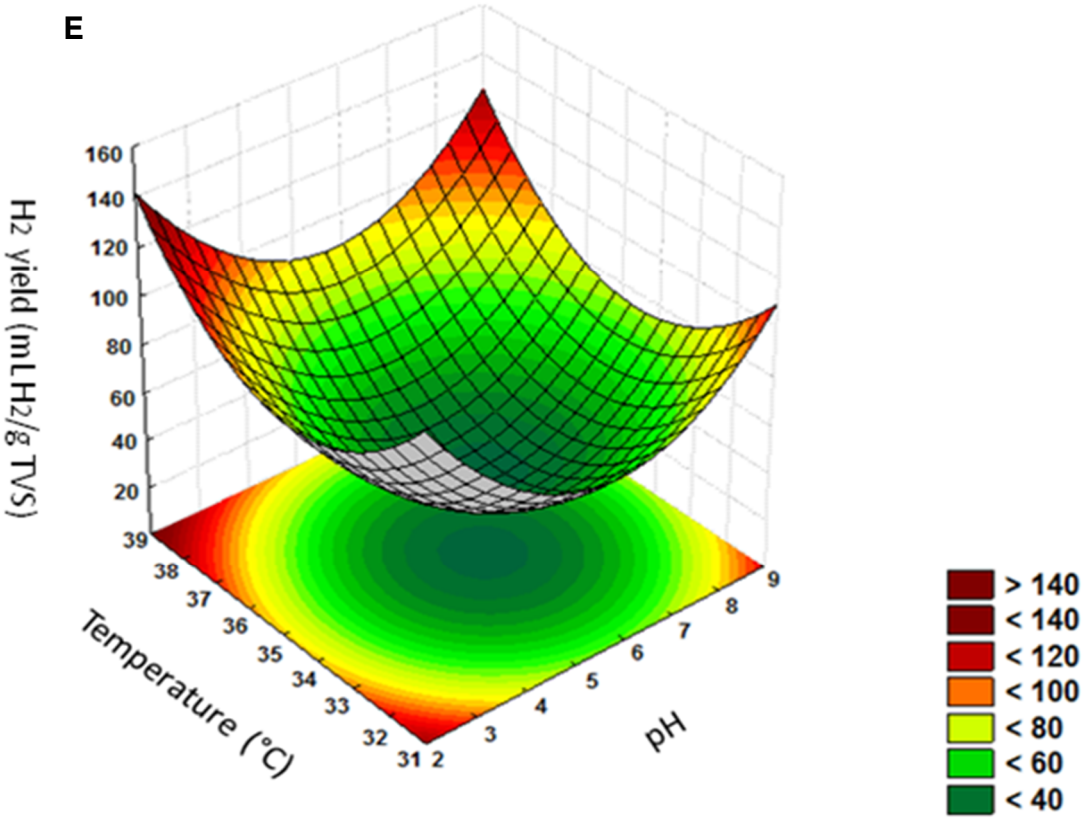
C


H2 yield (mL H2/g TVS)
120
100
90
80
70
60
50
40
30
20
pH
9
8
7
6
5
4
3
2
Time (h)
0
20
40
60
80
100
120
140
> 90
< 90
< 80
< 70
< 60
< 50
< 40

D


H2 yield (mL H2/g TVS)
100
90
80
70
60
50
40
30
20
Temperature (°C)
39
38
37
36
35
34
33
32
31
Time (h)
0
20
40
60
80
100
120
140
> 80
< 80
< 70
< 60
< 50
< 40

E


H2 yield (mL H2/g TVS)
160
140
120
100
80
60
40
20
Temperature (°C)
39
38
37
36
35
34
33
32
31
pH
2
3
4
5
6
7
8
9
> 140
< 140
< 120
< 100
< 80
< 60
< 40

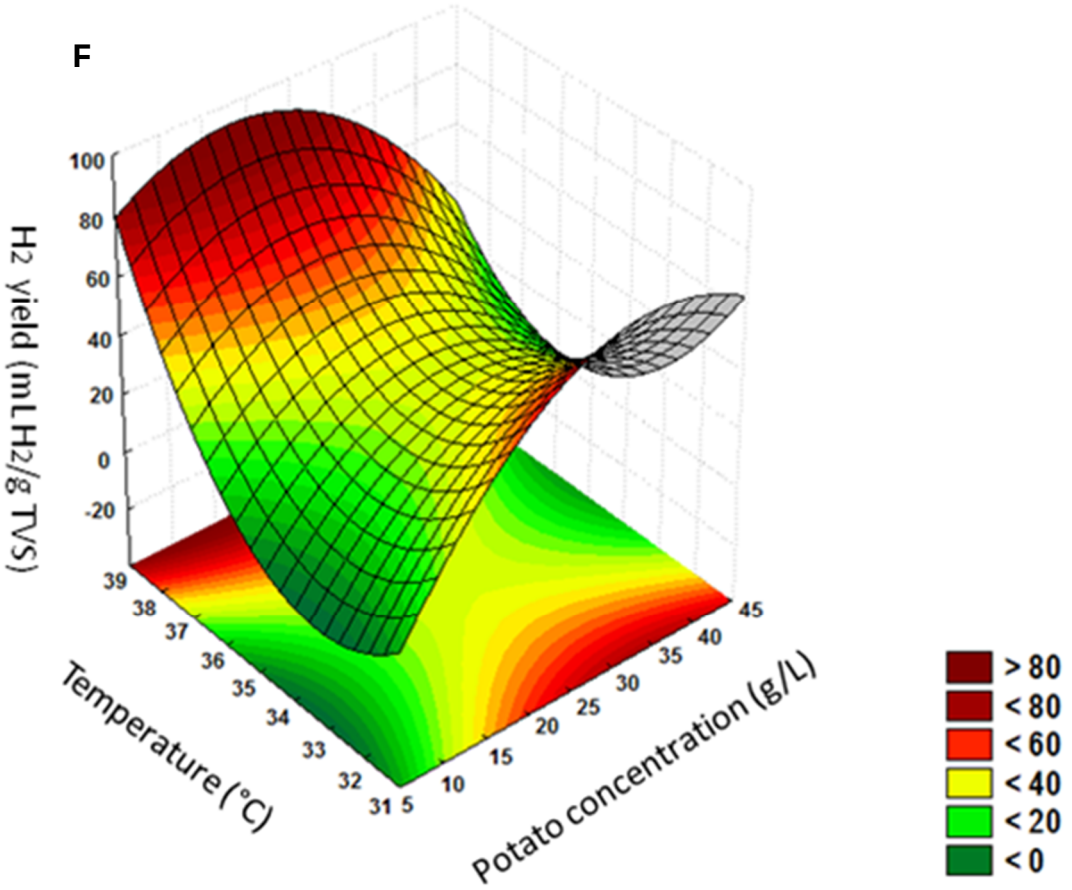


Figure 4.3 (A-F): Three-dimensional response surface plots showing the pairwise interaction between operating variables on biohydrogen production yield.

4.3.1.3 Optimization of operating variables

Having understood the effect of operating variables on biohydrogen yield, the process conditions were optimized. Equation 4.3 was solved according to the method of Myers and Montgomery (1995) to obtain the optimum values for the variables. The solution of Equation 4.3 were as follows: potato waste concentration, 39.56 g/L; fermentation time, 82.58 h; pH, 5.56; and temperature, 37.87 °C; with a corresponding biohydrogen yield of 68.54 mL H_2/g TVS. Furthermore, biohydrogen production was carried out at these optimized conditions for optimal biohydrogen production. Results from the experiments generated a biohydrogen yield of 79.43 mL H_2/g TVS showing an increase of 15.9% in the biohydrogen production yield.

4.3.2 Biohydrogen scale-up study

4.3.2.1 Biohydrogen production using optimum operating conditions

The scale-up study was conducted to evaluate the possibility of enhancing the yield of biohydrogen as a step towards its large-scale production using potato waste. The performance of the scale-up study was evaluated based on the cumulative biohydrogen production and the biohydrogen yield. The biogas obtained consisted mainly of biohydrogen and carbon dioxide (Figure 4.4 (A) and (B)) due to their stoichiometric relationship as shown in acetate and butyrate-fermentation reactions (see Equations 4.4 and 4.5). Biohydrogen production commenced after a short lag phase of 4 h and reached a fraction of 56.38% (Figure 4.4 (A)) and cumulative volume of 4820 mL (Figure 4.4 (B)) corresponding to a yield of 298.11 mL H_2/g TVS. There was no methane production during the fermentation process because the sludge was pretreated (90 ^{o}C for 30 minutes) to prevent the growth of methanogenic archaea which use biohydrogen for their metabolic processes (Faloye et al., 2013).

Biohydrogen production proceeds via a series of metabolic pathways and involves the transfer of protons and electrons. This process is facilitated by hydrogenase enzymes (Xia et al., 2016). There is high conversion during the exponential growth phase and this allows the hydrogenase enzymes to reach equilibrium and increase hydrogen production (Xia et al., 2016). Moreover, spore-germination occurs in predominant biohydrogen-producing bacteria (e.g. *Clostridium* species) and uses the substrate for their metabolic activity which in turn produces biohydrogen. The short lag phase attained in this study shows that bacterial cells adapted quickly to reactor conditions and were able to degrade the substrate (potato waste) due to its rich carbohydrate content (Wu and Lin, 2004). A decrease in biohydrogen fraction was observed from 46-84 h due to a switch in biochemical pathways from acidogenesis to solventogenesis (Wu and Lin, 2004).

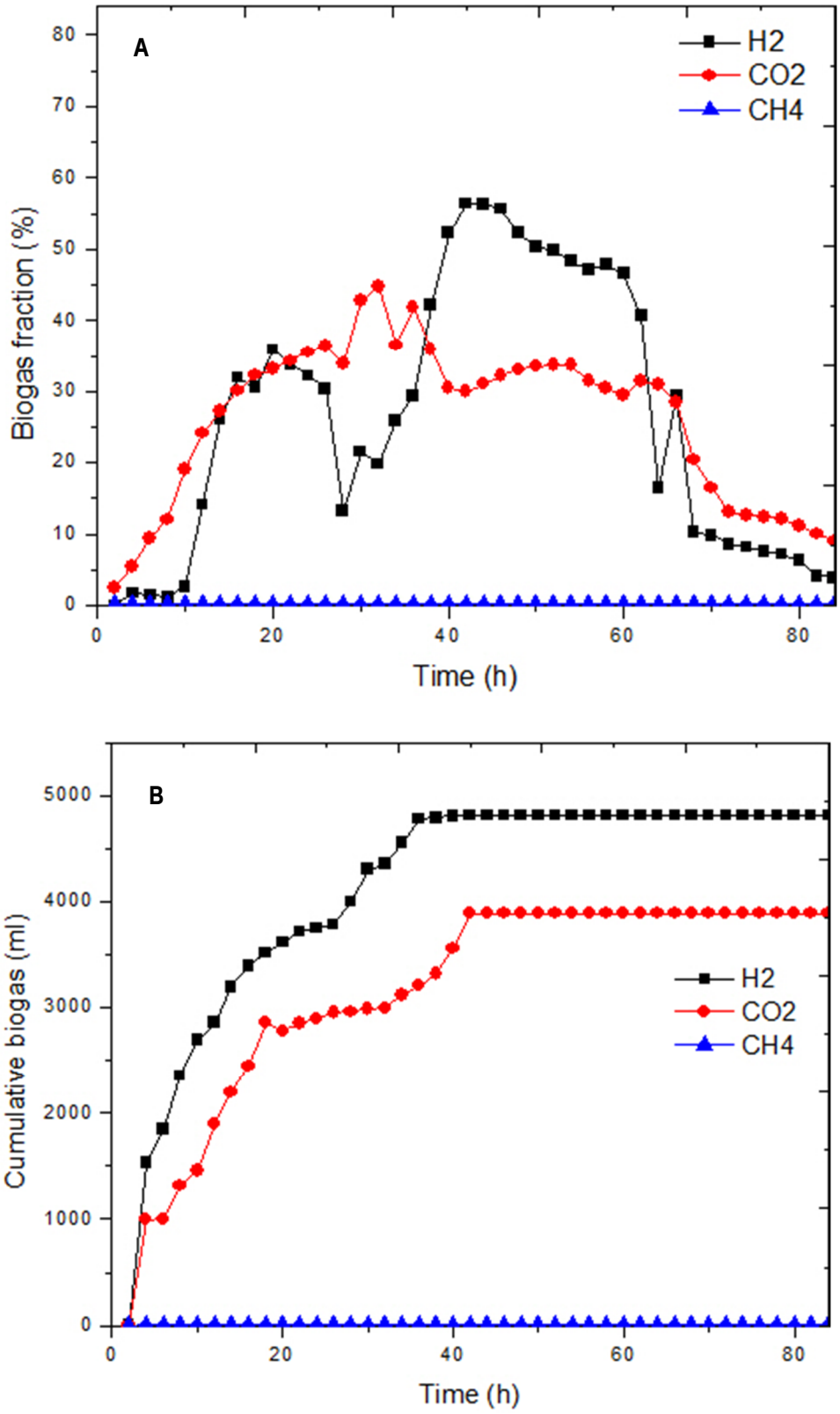


Figure 4.4: Biogas fraction (hydrogen, carbon dioxide, and methane) produced using immobilized anaerobic mixed bacteria (A) and the (B) cumulative biogas volume.

4.3.2.2 Volatile fatty acids production during biohydrogen production

Biohydrogen production predominantly occurs in the acidification/acidogenic stage where bacteria convert nutrients into biohydrogen. This causes the production of intermediate by-products such as acetate, butyrate, propionate, and ethanol (Sekoai and Gueguim Kana, 2013; Xia et al., 2016). These intermediates are crucial because they enable us to monitor the biohydrogen production trend (lag, exponential and death phase). To evaluate the production of volatile fatty acids (VFAs) during the fermentation process, broth samples were collected at 1 h interval from the scale-up reactor and analyzed for VFAs. The major VFAs detected were acetate, butyrate, and propionate (Figure 4.5), accounting for 62.89, 30.29, and 11.38%, respectively during the exponential growth-phase (42 h). This implies that the acetate-fermentation pathway was used by the biohydrogen-producing bacteria. This reaction is favoured by biohydrogen-producers because it increases the biohydrogen production yields as emphasized earlier (Xia et al., 2016). These results are consistent with stoichiometric relationship of Equation 4.4 and Equation 4.5; the theoretical yield is 4 mol H_2/mol glucose for acetate-reaction and 2 mol H_2/mol glucose for butyrate-reaction. However, it has been shown in some studies that the acetate-reaction does not always yields high biohydrogen because homoacetogens use this pathway for their metabolic activity (Saady, 2013). Metabolites such as propionate and ethanol are not suitable for biohydrogen production because they produce reactions that consume hydrogen as shown in Equations 4.6 and 4.7 (Sekoai and Gueguim Kana, 2013).

Acetate: $C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$ (4.4)

Butyrate: $C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$ (4.5)

Propionate: $C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O$ (4.6)

Ethanol: $C_6H_{12}O_6 \rightarrow CH_3CH_2OH + CO_2$ (4.7)

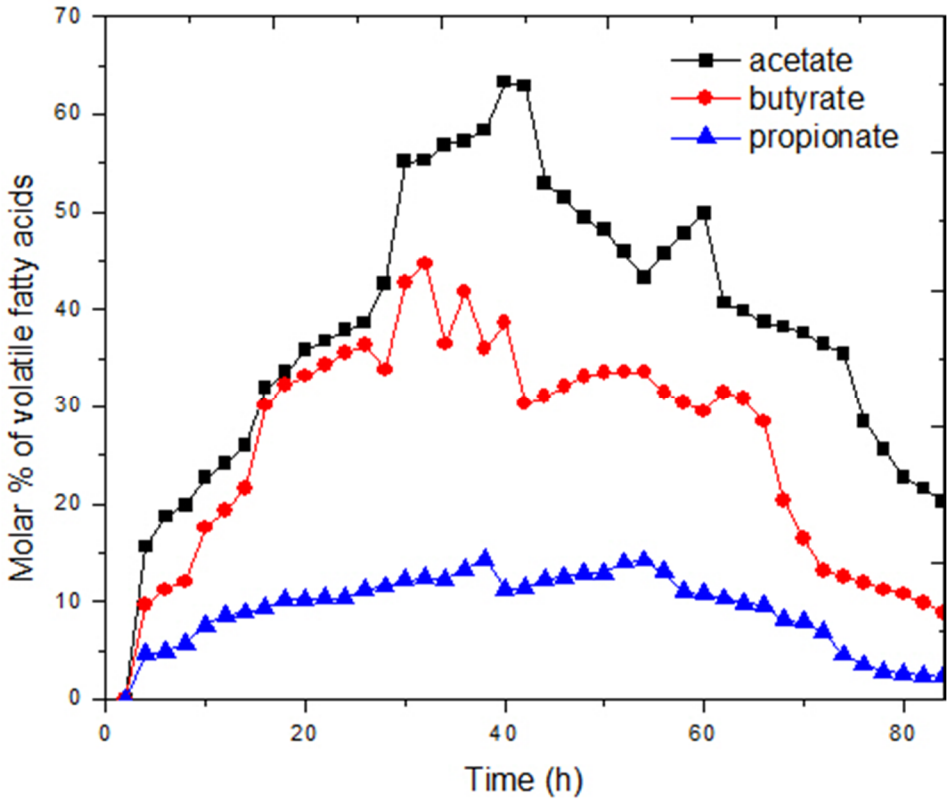


Figure 4.5: Volatile fatty acids produced during biohydrogen production.

4.3.2.3 pH profile during biohydrogen production

A gradual decrease in pH (5.56 to 3.58) was observed during biohydrogen production (Figure 4.6). This was attributed to the production of the abovementioned intermediates (VFAs) which reduce the buffering capacity of the medium during its production. This trend was also observed in other biohydrogen production studies (Elbeshbishy et al., 2011). Low pH values (less than 4) are not suitable for biohydrogen production because they decrease hydrogenase activity, extend the lag time, and disrupt the cell membrane (Xia et al., 2016). Therefore, it is important to control pH during biohydrogen production to prevent the activity of biohydrogen-inhibiting reactions while maintaining conditions that are suitable for hydrogen-producers (Xia et al., 2016). pH can be regulated at large-scale using dedicated sensors and actuators (Sekoai and Gueguim Kana, 2014).

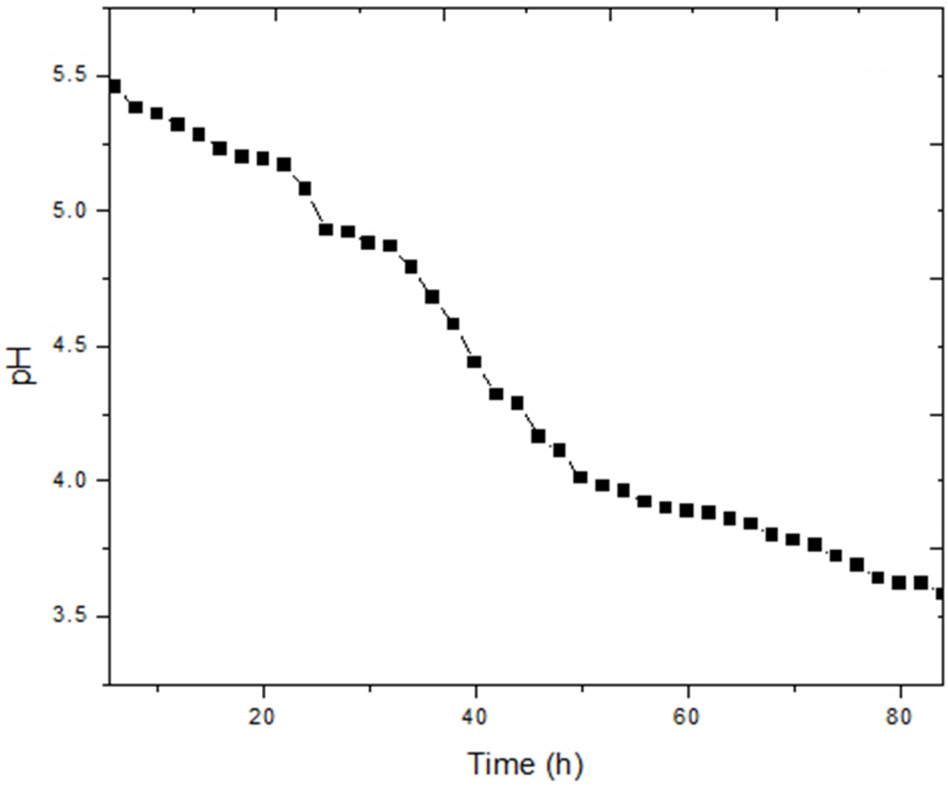


Figure 4.6: pH profile during biohydrogen production.

4.3.2.4 Morphological changes of alginate beads during fermentation process

Alginate beads were used as an encapsulating matrix for the inoculum (anaerobic sludge) during the scale-up study. Their physical and morphological changes were evaluated before and after the fermentation process as shown in Figures 4.7 and 4.8. The cells were still stable and reusable even after the fermentation process as shown in Figure 4.7 (B). Scanning electron microscope (SEM) images showed that the unused beads had a smooth inner surface (Figure 4.8 (A)), whereas the used beads were more porous (Figure 4.8 (B)) due to diffusion of nutrients during biohydrogen production. A similar observation was reported in biohydrogen studies that employed immobilized bacteria (Lin et al., 2006; Wu et al., 2005).

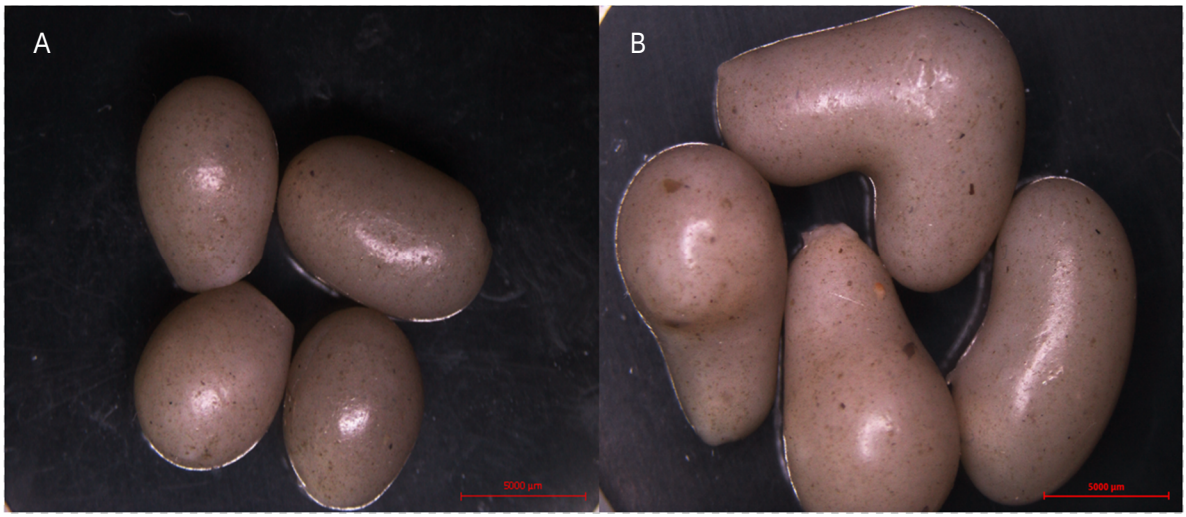


Figure 4.7: Stereomicroscopic images of calcium alginate immobilized beads before (A) and after (B) biohydrogen production.

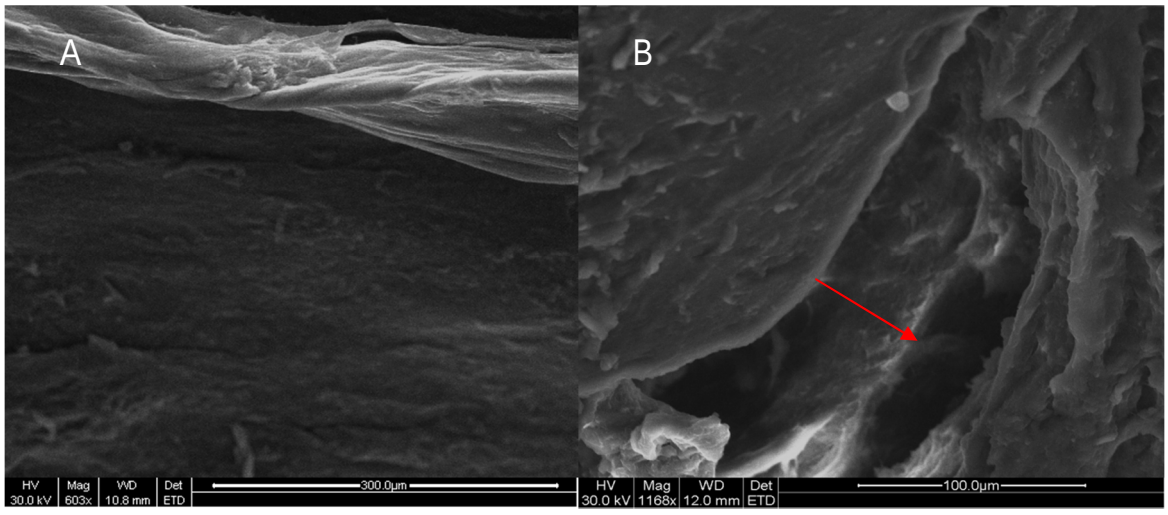


Figure 4.8: Scanning electron microscopic images of anaerobic mixed bacteria immobilized in calcium alginate solution before (A) and after (B) biohydrogen production. The arrow shows the porosity caused by diffusion of nutrients on alginate beads.

4.3.2.5 Scale-up studies: Immobilized cells versus suspended cells

The performance of this scale-up system was compared with that of suspended culture under similar operating conditions. The scale-up system which employed immobilized cells produced a biohydrogen yield of 298.1 mL H_2/g TVS, whereas a biohydrogen yield of 246.9

mL H_2/g TVS was obtained from suspended cells, indicating a 17.2% biohydrogen decrease. Therefore, utilization of immobilized cells could pave a way for large-scale biohydrogen production, and could help to overcome some of the challenges such as accumulation of oxygen in the reactor headspace, rapid drop in pH, contamination, and inconsistent mixing pattern faced by this process (Kumar et al., 2016; Sekoai et al., 2017).

4.4 Summary

In this chapter, results of the parametric optimization and scale-up study of biohydrogen production from potato waste are presented. Prior to the optimization study, the effect of operating variables (potato waste concentration, fermentation time, pH, and temperature) on biohydrogen yield was studied via response surface methodology approach using a 2^4-central composite design (CCD). The developed empirical model was used to explain the main and interaction effect of the aforementioned variables on biohydrogen yield. Furthermore, the model was solved to give the optimum operating conditions. The operating variables investigated in this study displayed significant influence on biohydrogen yield. Optimization of biohydrogen yield using the developed model generated the following optimum conditions: potato waste concentration 39.56 g/L; temperature 37.87 ^oC; pH 5.56; and fermentation time 82.68 h with a predicted yield of 68.54 mL H_2/g TVS. Scale-up study was conducted using immobilized technology with microbes immobilized on alginate matrix. The biohydrogen yield achieved using these optimized conditions was 79.43 mL H_2/g TVS, reflecting a 15.9% increase. The yield obtained for the scale-up study using the immobilization technology was 298.11 mL H_2/g TVS, while a biohydrogen yield of 246.9 mL H_2/g TVS was obtained from suspended cultures. Therefore, these results illustrate the potential of maximizing biohydrogen yield using response surface methodology approach along with immobilized cells, and could be instrumental to optimizing the process at large-scale production. The novel contributions described in this chapter have resulted in three

scientific manuscripts. This includes a review article that was published in "*Critical Reviews in Biotechnology"*. One experimental chapter has been published in "*Environments*", an international open access journal, and the second one is currently under review at "*Waste and Biomass Valorization*" (see Appendix A for copies of these papers).

References

Antonopoulou, G., Stamatelatou, K., Venetsaneas, N., Kornaros, M., and Lyberatos, G. (2008). Biohydrogen and methane production from cheese whey in a two-stage anaerobic process. Industrial Engineering and Chemical Research 47, 5227-5233.

Argun, H., Kargi, F., and Kapdan, I.K. (2008). Light fermentation of dark fermentation effluent for bio-hydrogen production by different *Rhodobacter* species at different initial volatile fatty acid (VFA) concentration. International Journal of Hydrogen Energy 33, 7405–7412.

Ayeni, A.O., Hymore, H.K., Mudliar, S.N., Deshmukh, S.C., Satpute, D.B., Omoleye, J.A., and Pandey, R.A. (2013). Hydrogen peroxide and lime based oxidative pretreatment of wood waste to enhance enzymatic hydrolysis for a biorefinery: Process parameters optimization using response surface methodology. Fuel 106, 187–94.

Boboescu, I.Z., Gherman, V.S., Lakatos, G., Pap, B., Biro, T., and Maroti, G. (2016). Surpassing the current limitations of biohydrogen production systems: The case for a novel hybrid approach. Bioresource Technology 204, 192-201.

Bundhoo, M.A.Z., and Mohee, R. (2016). Inhibition of dark fermentative bio-hydrogen production: A review. International Journal of Hydrogen Energy 41, 6713-6733.

Chong, M.L., Rahim, R.A., Shirai, Y., and Hassan, M.A. (2009). Biohydrogen production by *Clostridium butyricum* EB6 from palm oil mill effluent. International Journal of Hydrogen Energy 34, 746-771.

Dhillon, G.S., Brar, S.K., Verma, M., and Tygi, R.D. (2011). Apple pomace ultrafiltration sludge - A novel substrate for fungal bioproduction of citric acid: Optimisation studies. Food Chemistry 128, 864-871.

Duarte, J.C., Rodrigues, J.A., Moran, P.J.S., Valenca, G.P., and Nunhez, J.R. (2013). Effect of immobilized cells in calcium alginate beads in alcohol fermentation. AMB Express 3, 31.

Elbeshbishy, E., Hafez, H., Dhar, B.R., and Nakhla, G. (2011). Single and combined effect of various pretreatment methods for biohydrogen production from food waste. International Journal of Hydrogen Energy 36, 11379-87.

Faloye, F.D., Gueguim Kana, E.B., and Schmidt, S. (2013). Optimization of hybrid inoculum techniques for biohydrogen production and preliminary scale up. International Journal of Hydrogen Energy 38, 11765-11773.

Fan, Y., Li, C., Lay, J.L., Hou, H., and Zhang, G. (2004). Optimization of initial substrate and pH levels for germination of sporing hydrogen-producing anaerobes in cow dung compost. Bioresource Technology 91, 189-193.

Hallenbeck, P.C., and Ghosh, D. (2009). Advances in fermentative biohydrogen production: the way forward? Trends in Biotechnology 27, 287-97.

Keskin, T., Giusti, L., and Azbar, N. (2012). Continuous biohydrogen production in immobilized biofilm system versus suspened cell culture. International Journal of Hydrogen Energy 37, 1418-1424.

Kim, J.K., Oh, B.R., Chun, Y.N., and Kim, S.W. (2006). Effects of temperature and hydraulic retention time on anaerobic digestion of food waste. Journal of Biosciences and Bioengineering 102, 328-332.

Khanna, N., and Das, D. (2013). Biohydrogen production by dark fermentation. Wiley Interdisciplinary Reviews: Energy Environment 2, 401-421.

Kumar, G., Mudho, A., Sivagurunathan, P., Nagarajan, D., Ghimire, A., Lay, C.H., Lin, C.Y., Lee, D.J., and Chang, J.S. (2016). Recent insights into cell immobilization technology

applied for dark fermentation hydrogen production. Bioresource Technology 219, 725-737.

Leino, H., Kosourov, S.N., Saari, L., Sivonen, K., Tsygankov, A.A., Aro, E.M., and Allahverdiyeva, Y. (2012). Extended H_2 photoproduction by N_2-fixing cyanobacteria immobilized in thin alginate films. International Journal of Hydrogen Energy 30, 151–161.

Lin, C.N., Wu, S.Y., and Chang, J.S. (2006). Fermentative hydrogen production with a draft tube fluidized bed reactor containing silicone-gel-immobilized anaerobic sludge. International Journal of Hydrogen Energy 3, 2200–2210.

Mafuleka, S., and Gueguim Kana, E.B. (2015). Modelling and optimization of xylose and glucose production from Napier grass using hybrid pre-treatment techniques. Biomass Bioenergy 77, 200-208.

Martins, S.C.S., Martins, C.M., Fiúza, L.M.C.G., and Santaella, S.T. (2013). Immobilization of microbial cells: A promising tool for treatment of toxic pollutants in industrial wastewater. African Journal of Biotechnology 12, 4412-4418.

Moodley, P., and Gueguim Kana, E.B. (2015). Optimization of xylose and glucose production from sugarcane leaves (*Saccharum officinarum*) using hybrid pretreatment techniques and assessment for hydrogen generation at semi-pilot scale. International Journal of Hydrogen Energy 40, 3859–3867.

Moodley, P., and Gueguim Kana, E.B. (2017). Optimization of operational parameters for biohydrogen production from waste sugarcane leaves and semi-pilot scale process assessment. Bioresources 12, 2015-2030.

Mullai, P., Rene, E.R., and Sridevi, K. (2013). Biohydrogen production and kinetic modeling using sediment microorganisms of pichavaram mangroves, India. Biomedical Research International, 1-9.

Myers, R.H., and Montgomery, D.C. (1995). Response surface methodology: process and product optimization using designed experiments. John Wiley & Sons, USA.

Pan, J., Zhang, R.M., El-Mashad, H., Sun, H., and Ying, Y. (2008). Effect of food to microorganism ratio on biohydrogen production from food waste via anaerobic fermentation. International Journal of Hydrogen Energy 33, 6968-6975.

Qiu, C., Zheng, Y., Zheng, J., Liu, Y., Xie, C., and Sun, L. (2016). Mesophilic and Thermophilic Biohydrogen Production from Xylose at Various Initial pH and Substrate Concentrations with Microflora Community Analysis. Energy Fuels 30, 1013-1019.

Rorke, D., and Gueguim Kana, E.B. (2016). Biohydrogen process development on waste sorghum (*Sorghum bicolar*) leaves: Optimization of saccharafication, hydrogen and preliminary scale up. International Journal of Hydrogen Energy 41, 12941-12952.

Saady, N.M.C. (2013). Homoacetogenesis during hydrogen production by mixed cultures dark fermentation. International Journal of Hydrogen Energy 38, 13172-13191.

Saleh, A.F., Kamarudin, E., Yaacob, A.B., Yussof, A.W., and Abdullah, M.A. (2012). Optimization of biomethane production by anaerobic digestion of palm oil mill effluent using response surface methodology. Asia-Pacific Journal of Chemical Engineering 7, 353–360.

Sekoai, P.T. (2016). Modelling and Optimization of Operational Setpoint Parameters for Maximum Fermentative Biohydrogen Production Using Box-Behnken Design. Fermentation 2, 1-11.

Sekoai, P.T., and Gueguim Kana, E.B. (2013). A two-stage modelling and optimization of biohydrogen production from a mixture of agro-municipal waste. International Journal of Hydrogen Energy 38, 8657-8663.

Sekoai, P.T., and Gueguim Kana, E.B. (2014). Semi-pilot scale production of hydrogen from Organic Fraction of Solid Municipal Waste and electricity generation from process effluents. Biomass and Bioenergy 60, 156-163.

Sekoai, P.T., Awosusi, A., Yoro, K.O., Singo, M., Oloye, O., Ayeni, A., Bodunrin, M., and Daramola, M. (2017). Microbial cell immobilization in biohydrogen production - A short overview. Critical Reviews in Biotechnology, 1-15.

Sekoai, P.T., Yoro, K.O., and Daramola, M.O. (2016). Batch fermentative biohydrogen production process using immobilized anaerobic sludge from organic solid waste. Environments 3, 1-10.

Show, K.Y., Lee, D.J., Tay, J.H., Lin, C.Y., and Chang, J.S. (2012). Biohydrogen production: Current perspectives and the way forward. International Journal of Hydrogen Energy 37, 15616-15631.

Sinha, P., and Pandey, A. (2011). An evaluative report and challenges for fermentative biohydrogen production. International Journal of Hydrogen Energy 36, 7460-7478.

Urak, S., Yeniay, O., and Karasu-Yalcin, S. (2015). Optimization of citric acid production from a carrot juice-based medium by *Yarrowia lipolytica* using response surface methodology. Annuals in Microbiology 65, 639–649.

Wang, J.L., and Wan, W. (2008). Effect of temperature on fermentative hydrogen production by mixed cultures. International Journal of Hydrogen Energy 33, 5392–5397.

Wong, Y.M., Wu, T.Y., and Juan, J.C. (2014). A review of sustainable hydrogen production using sludge via dark fermentation. Renewable and Sustainable Energy Reviews 34, 471-482.

Wu, J.H., and Lin, C.Y. (2004). Biohydrogen production by mesophilic fermentation of food wastewater. Water Science and Technology 49, 223-228.

Wu, S.Y., Lin, C.N., and Chang, J.S. (2005). Biohydrogen production with anaerobic sludge immobilized by ethylene-vinyl acetate copolymer. International Journal of Hydrogen Energy 30, 1375–1381.

Xia, A., Jacob, A., Hermann, C., and Murphy, D. (2016). Fermentative bio-hydrogen production from galactose. Energy 96, 346-354.

Yaakob, H., Ahmed, R.N., Daud, S.K., Malek, R.A., and Rahman, R.A. (2012). Optimization of Ingredient and Processing Levels for the Production of Coconut Yogurt Using Response Surface Methodology. Food Science and Biotechnology 21, 933-940.

Chapter 5 – Effect of metal ions on dark fermentative biohydrogen production using suspended and immobilized cells of anaerobic mixed bacteria

The results of the investigation of the effect of metal ions on dark fermentative biohydrogen production performance using suspended and immobilized anaerobic mixed bacteria are reported in this chapter. As a result of insufficient data in literature, this study was therefore undertaken to provide insights on the effect of metal ions on biohydrogen production using immobilized cells.

5.1 Introduction

Evaluating the functional role of metal ions during dark fermentative biohydrogen production is necessary because these supplementary nutrients play a key role in the metabolism of biohydrogen-producing bacteria. They stimulate the activity of hydrogenase enzymes and used for microbial growth (Srikanth and Venkata Mohan, 2012). Studies that have assessed the effect of metal ions such as iron (Fe^{2+}), calcium (Ca^{2+}), copper (Cu^{2+}), zinc (Zn^{2+}), nickel (Ni^{2}), and magnesium (Mg^{2+}) reported a remarkable improvement on biohydrogen production (Boni et al., 2014; Ding et al., 2004; Lee et al., 2001; Lin and Shei, 2008; Liu and Shen, 2004; Wang and Wan, 2008; Yang and Shen, 2006; Zhang et al., 2005; Zheng and Yu, 2005).

Nonetheless, the dark fermentation process is still plagued with low yields due to its complexities. The highest yield obtained using metal ions was 2.73 mol H_2/mol glucose and is about 68% of the theoretical value (Zhang et al., 2005). This necessitates a search for other novel biohydrogen enhancement methods. Recently, there has been an upsurge of interest in the utilization of immobilized bacteria in biohydrogen process development because these biocatalysts possess several merits such as high substrate conversion efficiency, high metabolic activity, shortened lag phase, increased cell density, easier handling, reusability,

better solid/liquid separation efficiency and better operational stability (Kourkoutas et al., 2004). Moreover, this technology can be incorporated in biohydrogen-producing reactors such as continuous stirred tank reactor (Kourkoutas et al., 2004), fluidized bed reactor (Lin et al., 2006), carrier induced granular sludge bed reactors (Zheng et al., 2009), up-flow anaerobic sludge bed reactors (Argun et al., 2008) and trickling biofilters (Eroglu et al., 2009).

Table 5.1 summarizes various studies in literature that have evaluated the effects of metal ions on biohydrogen production performance using suspended and immobilized cells. In these studies, different biohydrogen yields were obtained due to several contributing factors such as the type of substrate used, type of inoculum used, operating conditions, and metal ion concentration as shown in Table 5.1. It can be seen that studies using metal ions and immobilized cells are still scarce in literature. Better understanding of the effect of metal ions on biohydrogen production especially using immobilized cells could be instrumental to optimizing and up-scaling the process. Against this background, this chapter contains the results of the investigation conducted on the effect of metal ions (Fe^{2+}, Ca^{2+}, Mg^{2+} and Ni^{2+}) on batch fermentative biohydrogen production using suspended and immobilized cells of anaerobic mixed bacteria.

Table 5.1: Biohydrogen production studies that evaluated the effect of metal ions using suspended and immobilized cells.

Metal ion	Bacteria	Substrate	Suspended/Immobilized	Concentration range (mg/L)	Maximum H_2 yield	Reference
Fe^{2+}	Anaerobic sludge	Glucose	Suspended cells	0-1500	334.2[e]	Wang and Wan (2008)
	Clostridium butyricum EB6	Glucose	Suspended cells	150-450	2.2[a]	Chong et al. (2009)
	Mixed sludge	Sucrose	Suspended cells	0-4000	24[b]	Lee et al. (2001)
	Dairy effluent bacteria	Dairy effluent	Suspended cells	50-300	85[c]	Paul et al. (2014)
	Anaerobic sludge	Glucose	Suspended cells	0-200	19.29[d]	Srikanth and Venkata Mohan (2012)
	Anaerobic sludge	Starch	Suspended cells	0-201	274[e]	Yang and Shen (2006)
	Anaerobic sludge	Glucose	Suspended cells	1-8	41.6[f]	Lee et al. (2009)
	Mixed sludge	Sucrose	Suspended cells	0-1600	2.73[a]	Zhang et al. (2005)
	Clostridium sp. LS2	Palm oil effluent	Immobilized cells	100-400	7.3[b]	Singh and Wahid (2014)
Mg^{2+}	Anaerobic sewage sludge	Sucrose	Suspended cells	8-200	3.43[a]	Lin and Lay (2005)
	Anaerobic sludge	Glucose	Suspended cells	0-200	16.42[d]	Srikanth and Venkata Mohan (2012)
	Seed sludge	Glucose	Suspended cells	0-5000	124.78[c]	Wongtanet and Prapagdee (2008)
	Bacillus sp.	Starch	Suspended cells	20	1.19[a]	Bao et al. (2013)
Ca^{2+}	Anaerobic sewage sludge	Sucrose	Suspended cells	0-300	3.6[a]	Chang and Lin (2006)
	Clostridium acetobutylicum	Glucose	Suspended cells	0-272	391[e]	Alshiyab et al. (2008)
Ni^{2+}	Anaerobic sludge	Glucose	Suspended cells	0.5-50	1.4[a]	Karadag and Puhakka (2008)
	Anaerobic mixed sludge	Glucose	Suspended cells	0-64	14.8[d]	Srikanth and Venkata Mohan (2012)
	Anaerobic digested sludge	Glucose	Suspended cells	0-50	296.1[e]	Wang and Wan (2008)

[a] mol/mol substrate
[b] mL/g substrate/h
[c] mL
[d] mol/kg $COD_{removed}$
[e] mL/g substrate
[f] L/day

5.2 Materials and methods

5.2.1 Substrate and inoculum preparation

The potato waste was prepared using the procedure outlined in section 3.2.1. The anaerobic mixed consortium was heat-treated as described in section 3.2.2. Furthermore, it was immobilized using the protocol provided in section 4.2.5.1.

5.2.2 Batch fermentation experiments

In this study, $FeCl_2$ served as Fe^{2+} source, $MgCl_2$ as Mg^{2+} source, $CaCl_2$ as Ca^{2+} source, and $NiCl_2$ as Ni^{2+} source. Batch fermentation experiments were performed using modified 1 L Erlenmeyer flask reactors. Each experimental reactor was fed with 50 mL of pretreated sludge, 225 mL medium consisting of potato waste (39.56 g/L) and nutrient solution, and 225 mL of metal ion (Fe^{2+}, Ni^{2+}, Mg^{2+} or Ca^{2+}) at varying concentrations (0, 100, 300, 500, and 1000 mg/L). The nutrient solution consisted of the following (g/L): NH_4HCO_3 2.0, NH_4Cl 0.5, $Na_2MoO_4.2H_2O$ 0.025, KH_2PO_4 0.25, K_2HPO_4 0.25, $ZnCl_2$ 0.0115, $CuCl_2$ 0.0105, $MnSO_4$ 0.005 and $MnCl_2$ 0.015. The batch reactors were purged with nitrogen gas for 5 minutes and immediately sealed with silicone rubber stoppers to create anaerobic conditions suitable for biohydrogen production. The experiments were conducted in duplicate for accuracy of data and reduction in experimental error. The operating conditions were 5.56, 37.87 ^oC, and 82.58 hours for pH, temperature and fermentation time, respectively. These were established from the parametric optimization stage conducted in chapter 4. Experiments were carried out using a temperature-regulated stirring hot-plate at agitation speed of 100 rpm. In batch experiments using immobilized bacteria, the reactors were fed with alginate beads (total weight 130 g) (Figure 5.1) and the abovementioned support media. The operating conditions were kept the same as in the suspended cultures. A schematic representation of the set-up employed for biohydrogen production experiments is shown in Figure 5.2.

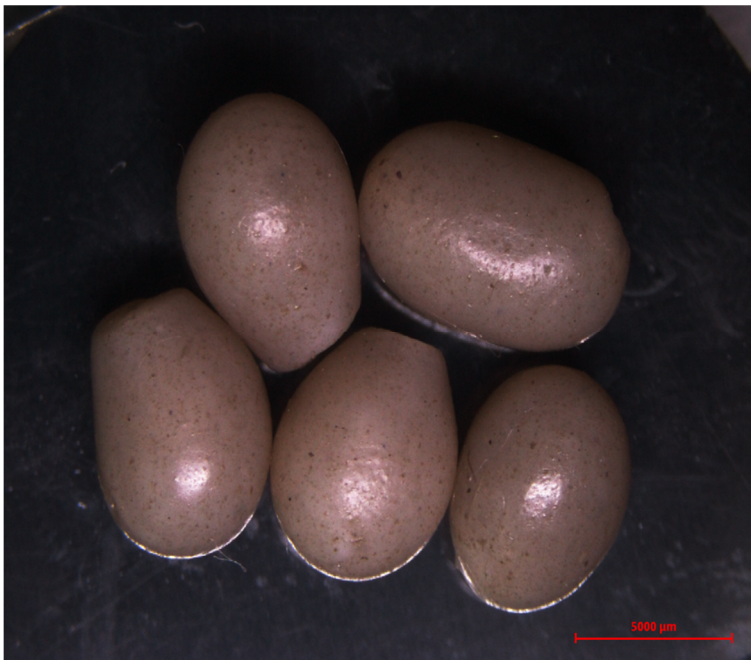


Figure 5.1: Morphology of the alginate beads employed for biohydrogen production.

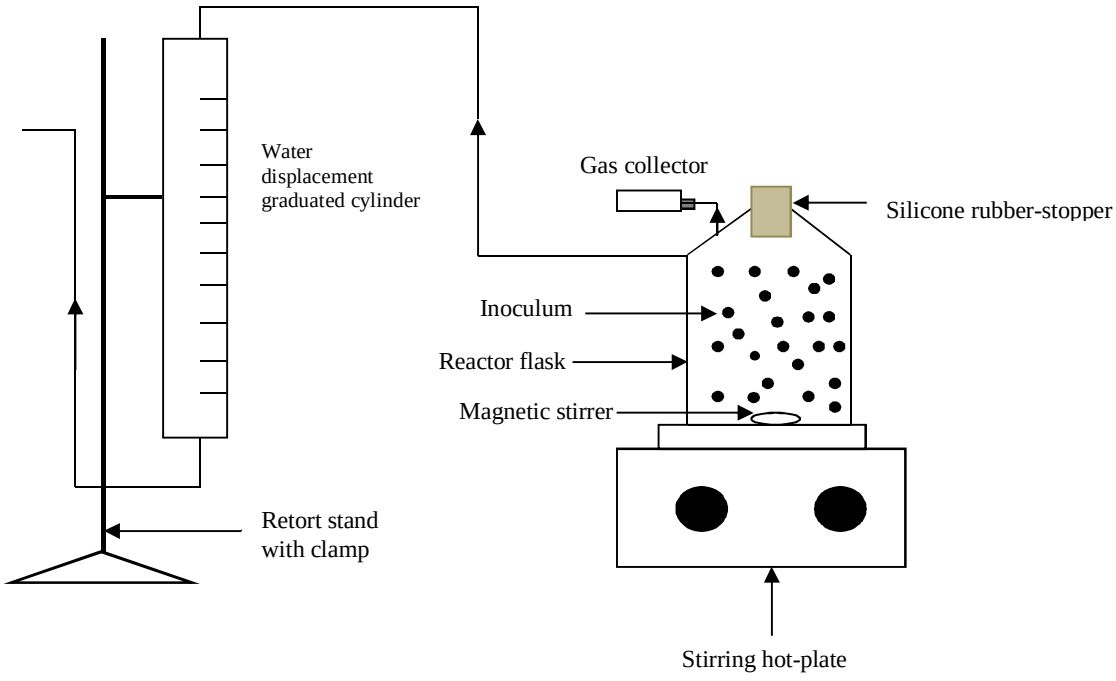


Figure 5.2: Biohydrogen production experimental set-up.

5.2.3 Analytical methods

The pH, biohydrogen fraction, cumulative biohydrogen, chemical oxygen demand (COD), and total volatile solids (TVS) were determined as described in section 3.2.6. Meanwhile, the physical and scanning electron microscopy (SEM) analysis of alginate beads was conducted using the detailed procedures in section 4.2.5.4.

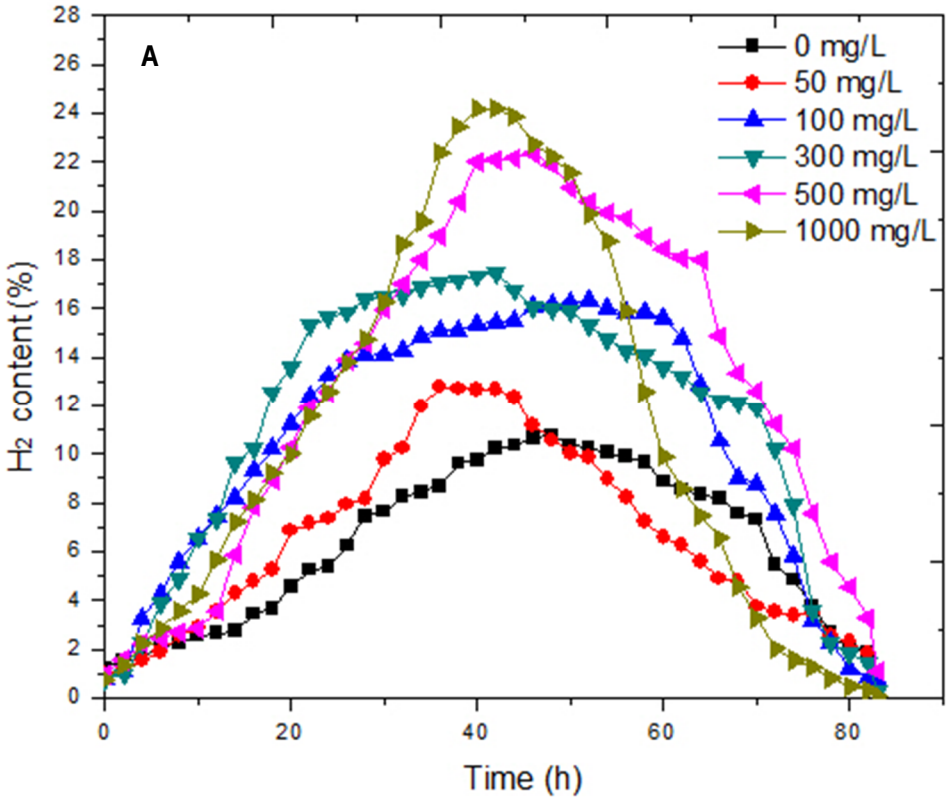
5.3 Results and discussion

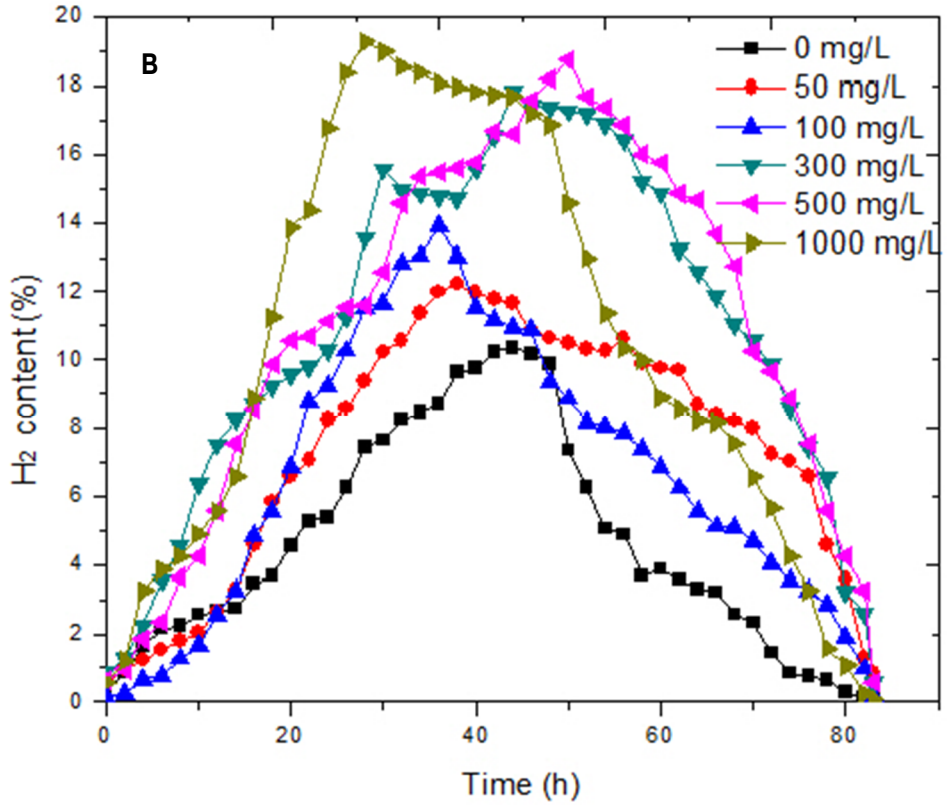
5.3.1 Effect of metal ions on biohydrogen production using suspended cells

Figures 5.3 and 5.4 illustrate the effect of metal ion concentrations (0-1000 mg/L) of Fe^{2+}, Mg^{2+}, Ca^{2+} and Ni^{2+}, on biohydrogen production using suspended cultures of anaerobic mixed sludge. Fe^{2+} and Mg^{2+} demonstrated a better biohydrogen production performance at 1000 mg/L. In these investigations, biohydrogen fractions of 24.2 and 19.3% were obtained at 1000 mg/L as shown in Figures 5.3 (A) and (B), respectively, corresponding to a cumulative volume of 2871 and 1231 mL, respectively (Figure 5.4 (A) and (B)). Meanwhile, a peak biohydrogen fraction of 16.3 and 15.6% (Figure 5.3 (C) and (D)) corresponding to cumulative volume of 356 and 185 mL (Figure 5.4 (C) and (D)) were produced in batch experiments using Ca^{2+} (1000 mg/L) and Ni^{2+} (1000 mg/L), respectively. The biohydrogen yield for the studied metals was as follows: Fe^{2+} (218.9 mL H_2/g TVS), Mg^{2+} (213.5 mL H_2/g TVS), Ca^{2+} (208.9 mL H_2/g TVS), and Ni^{2+} (202.3 mL H_2/g TVS), respectively.

These results are consistent with literature. Boni et al. (2014) reported a two-fold increment in biohydrogen production at high Fe^{2+} concentration of 1000 mg/L using suspended cultures. Karadag and Puhakka (2008) reported a 71% increase in biohydrogen production at 100 mg/L for Fe^{2+} and Ni^{2+}, respectively, using suspended cells. It can therefore be concluded that the presence of metal ions stimulates the activity of biohydrogen-producing microorganisms and enhances its yield when used at optimum concentrations (Karadag and Puhakka, 2008).

In contrast, lower biohydrogen production was achieved in batch experiments using 0 mg/L of metal ions as shown in Figure 5.3. A similar observation was confirmed by Zhu et al. (2007), where biohydrogen production by *Rhodobacter sphaeroides* was significantly suppressed when Fe^{2+} was limited (0 mg/L). Whereas the authors recorded a linearly increase in biohydrogen production at Fe^{2+} concentration of 0-1.6 mg/L.


A
0 mg/L
50 mg/L
100 mg/L
300 mg/L
500 mg/L
1000 mg/L
H2 content (%)
Time (h)


B
0 mg/L
50 mg/L
100 mg/L
300 mg/L
500 mg/L
1000 mg/L
H2 content (%)
Time (h)

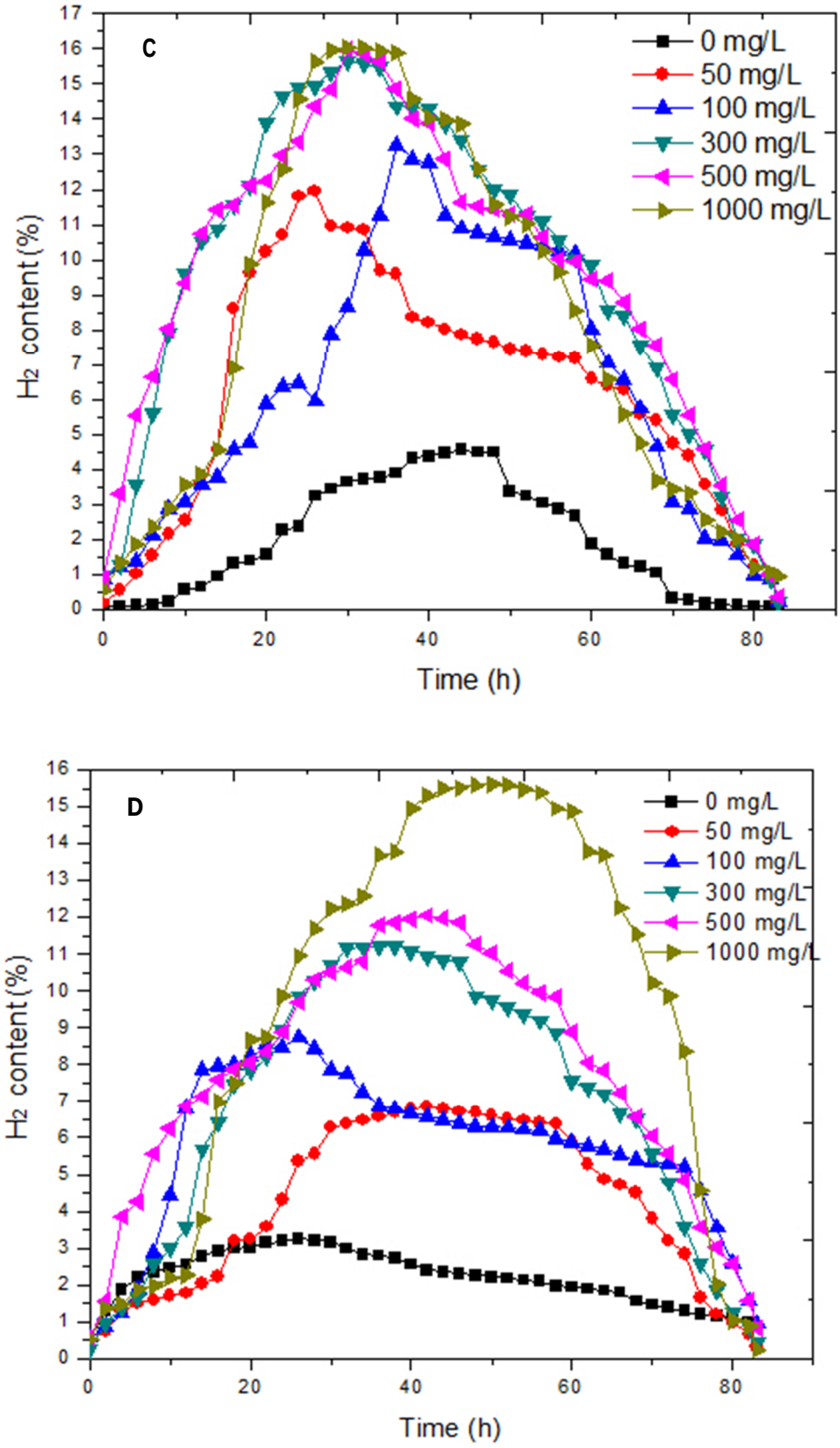
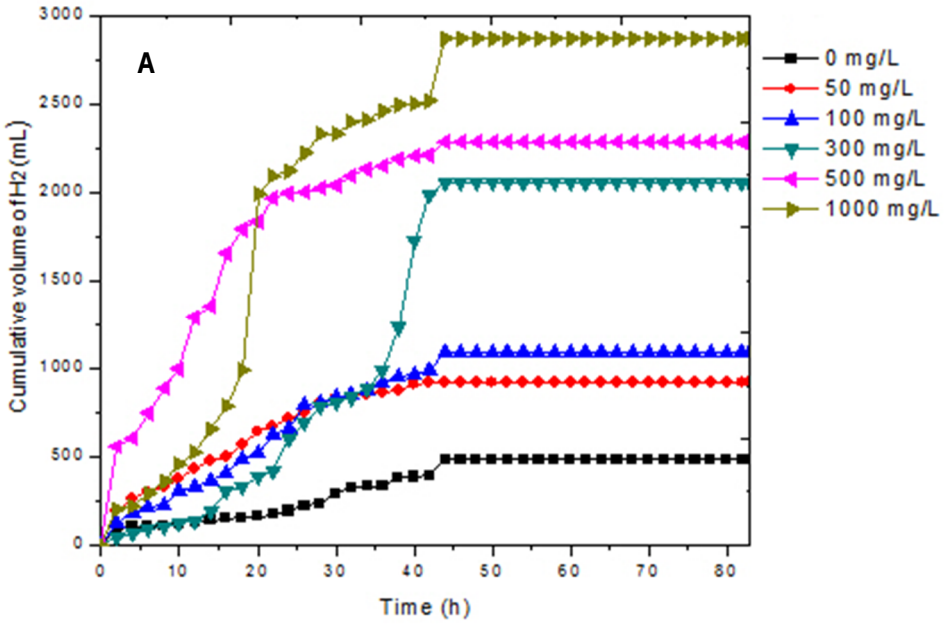
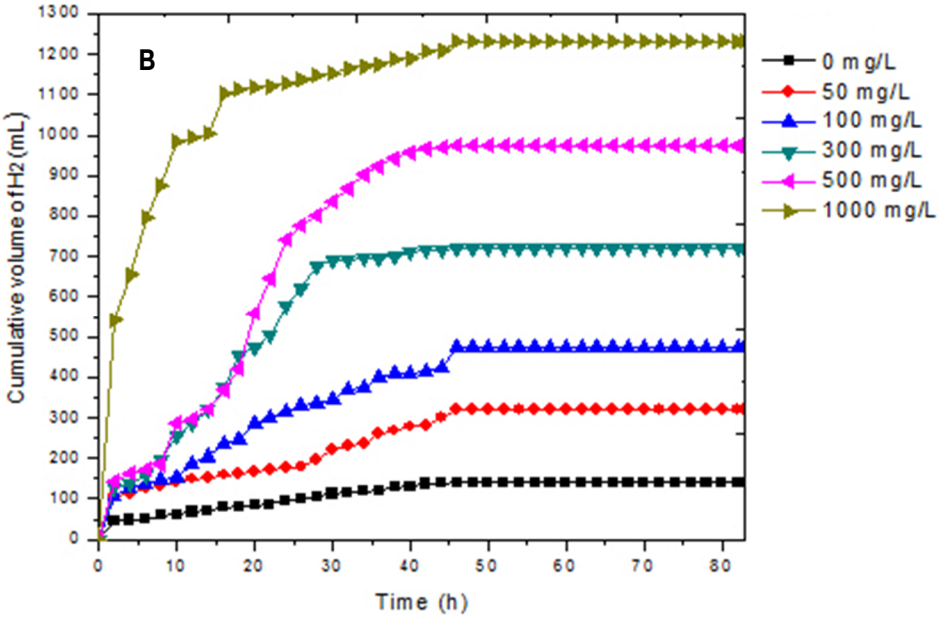


Figure 5.3: Effect of concentration (0-1000 mg/L) of Fe^{2+} (A), Mg^{2+} (B), Ca^{2+} (C) and Ni^{2+} (D) ion on biohydrogen production using suspended cells.


A
Cumulative volume of H2 (mL)
Time (h)
0 mg/L
50 mg/L
100 mg/L
300 mg/L
500 mg/L
1000 mg/L


B
Cumulative volume of H2 (mL)
Time (h)
0 mg/L
50 mg/L
100 mg/L
300 mg/L
500 mg/L
1000 mg/L

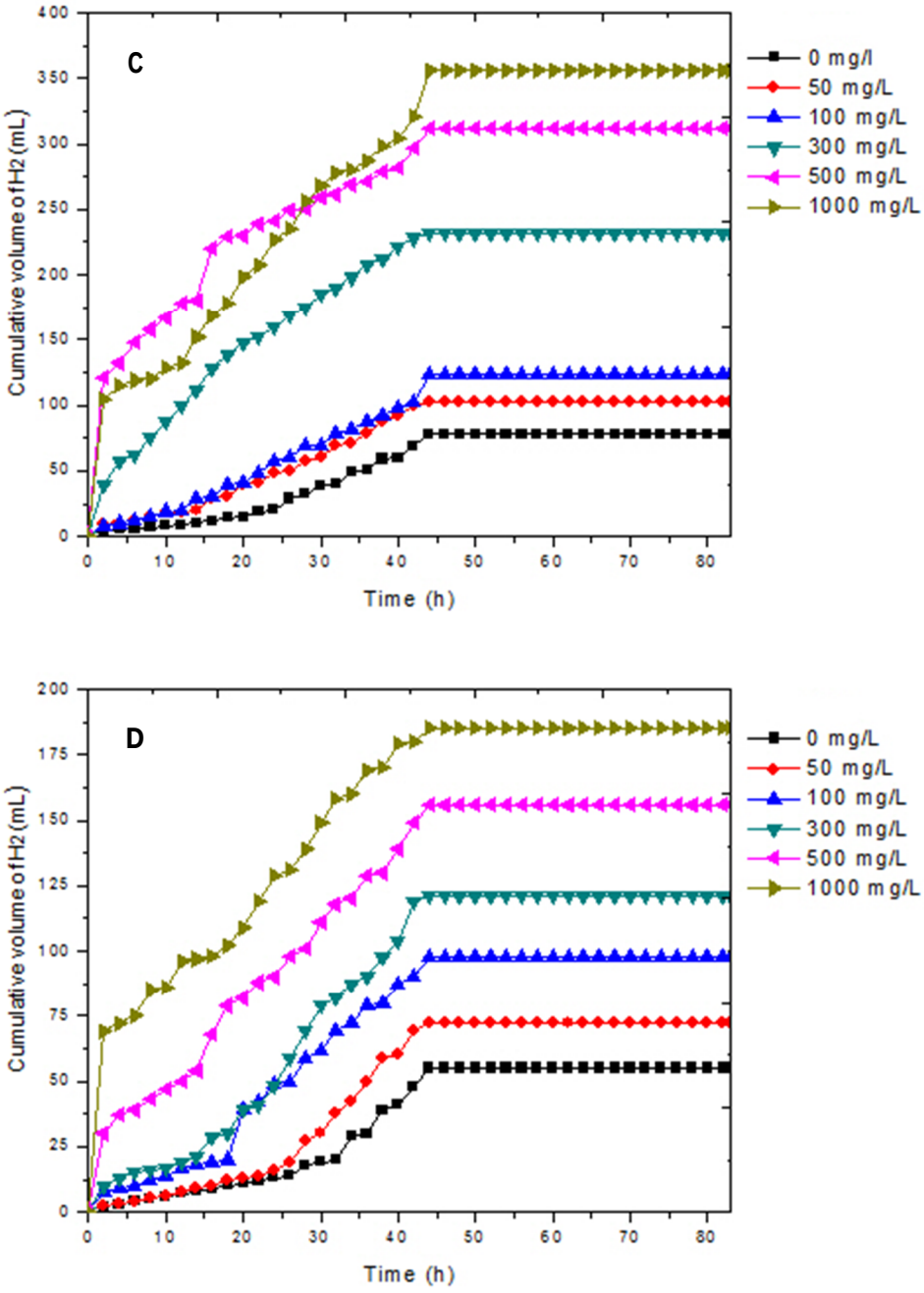


Figure 5.4: Effect of concentration (0-1000 mg/L) of Fe^{2+} (A), Mg^{2+} (B), Ca^{2+} (C) and Ni^{2+} (D) ion on cumulative biohydrogen production using suspended cells.

5.3.2 Effect of metal ions on biohydrogen production using immobilized cells

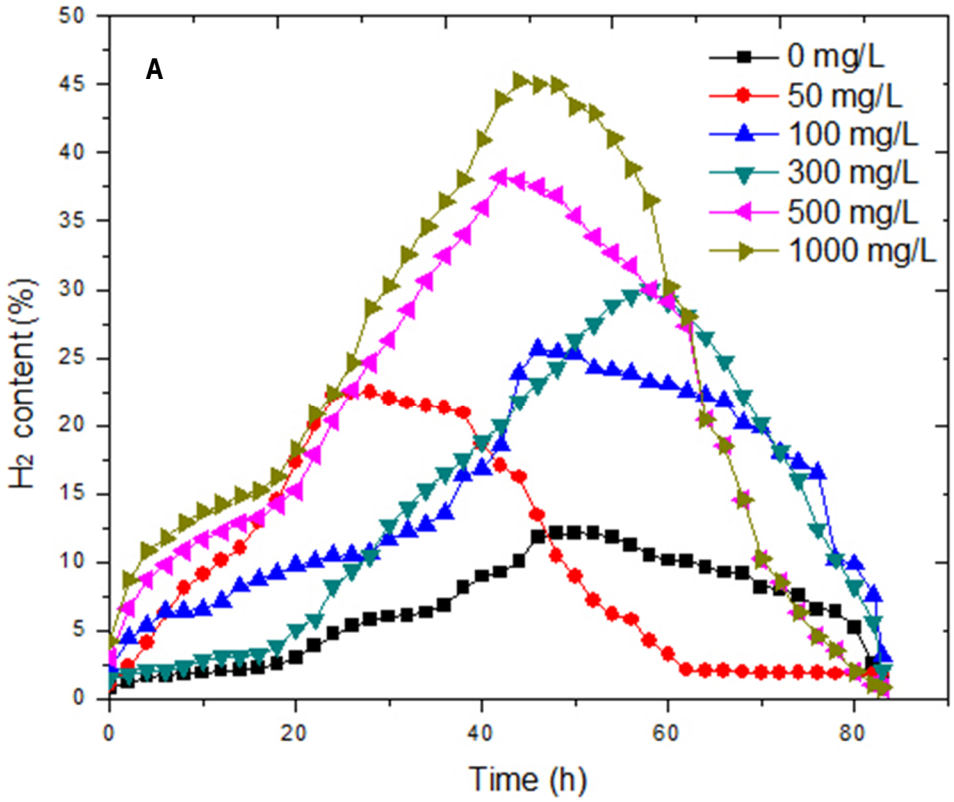
There are few studies in literature that have examined the effects of metal ions on biohydrogen production using immobilized bacteria. As far as could be ascertained, only the work of Singh and Wahid (2014) reported the effect of metal ions (Fe^{2+}, Mg^{2+}, K^{2+} and Ni^{2+}) on biohydrogen production using immobilized cells of *Clostridium* sp. LS2. Thus, there is a need for an in-depth understanding of the effects of metal ions on biohydrogen production using immobilized bacteria. Figure 5.5 (A – D) shows the effect of metal ions (Fe^{2+}, Mg^{2+}, Ca^{2+} and Ni^{2+}) on biohydrogen production using immobilized cells of anaerobic mixed bacteria. The use of immobilized cells showed a significant increase in biohydrogen production compared to suspended cells, particularly when using Fe^{2+} ions. Biohydrogen production increased from 12.12 (2 h) to 45.21% (44 h) when the concentration of Fe^{2+} increased from 0 to 1000 mg/L as indicated in Figure 5.5 (A). This corresponds to a cumulative volume of 4986 mL (Figure 5.6 (A)) and a biohydrogen yield of 292.8 mL H_2/g TVS. This value was 1.3 times higher than that of the suspended cultures. The observed increase in biohydrogen production can likely be attributed to the synergistic effect of cell immobilization and Fe^{2+}. Cell immobilization possesses several advantages such as protection of microorganisms against undesirable fermentative metabolites (e.g. volatile fatty acids and alcohols) which are synthesized during a switch in biochemical pathways from acidogenesis to solventogenesis (Kourkoutas et al., 2004). In addition, it enables bacteria to withstand low pH because they are not in direct contact with the fermentation medium and therefore extends their life span (Lin et al., 2006).

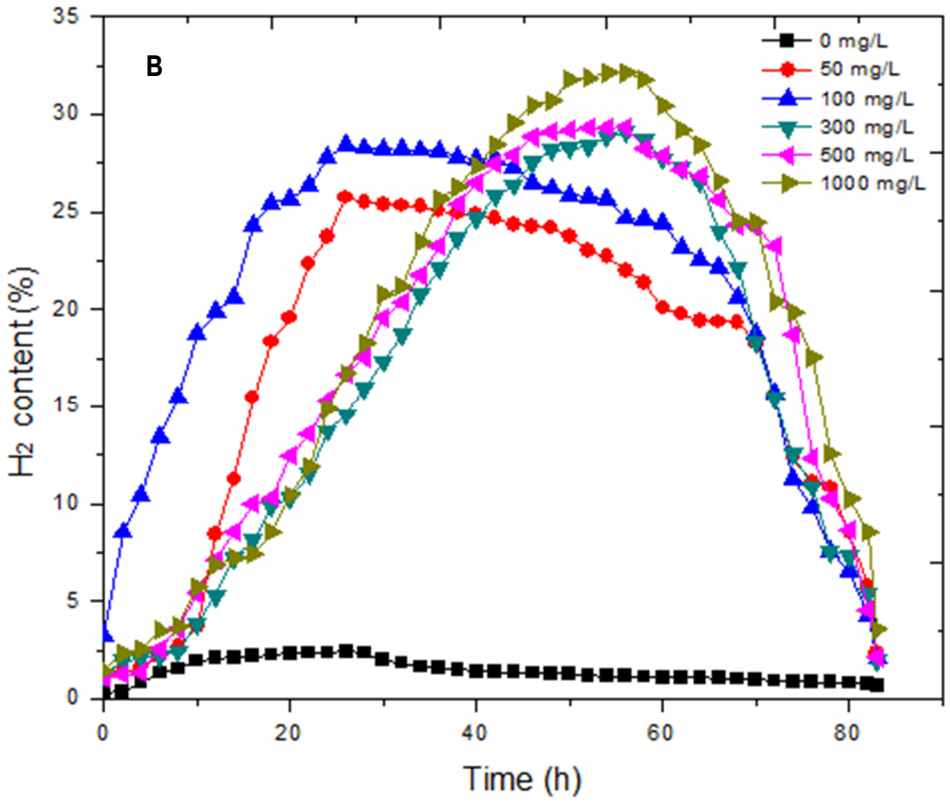
Iron (Fe^{2+}) is one of the most important metals required during dark fermentation process because it is actively involved in the synthesis of ferredoxin which is a key protein used for transfer of electrons in biohydrogen-producing bacteria such as *Clostridium* species (Lee et al., 2001). Singh and Wahid (2014) reported an improved biohydrogen production rate (289-

498 mL H_2/L.POME/h) when the concentration of Fe^{2+} was increased from 100 to 300 mg/L using immobilized *Clostridium* sp. LS2 cells. Duran-Padilla et al. (2014) studied the growth of biohydrogen-producing strain of *Clostridium acetobutylicum* ATCC 824 and observed that addition of Fe^{2+} (20 mg/L) enhanced its growth. Lee et al. (2009) evaluated the effect of Fe^{2+} on continuous biohydrogen production using a submerged membrane reactor, and concluded that Fe^{2+} is an essential component for biohydrogen-producing pathways because it increases the hydrogenase activity i.e. enzymes involved in the synthesis of molecular hydrogen (Lee et al., 2009). Moreover, it was reported in some studies that an increase in Fe^{2+} concentration favours the formation of biohydrogen-producing acetate and butyrate fermentation reactions (Duran-Padilla et al., 2014; Lee et al., 2001).

A similar biohydrogen production pattern (H_2 increased with increasing metal concentration) was observed when the concentration of Mg^{2+}, Ca^{2+} and Ni^{2+} ions was varied from 0 to 1000 mg/L. Biohydrogen production started after a short lag phase of 1, 2, and 4 h; and reached an optimum value of 32.12% at 1000 mg/L (Figure 5.5 (B)), 27.68% at 100 mg/L (Figure 5.5 (C)), and 22.78% at 500 mg/L (Figure 5.5 (D)); for Mg^{2+}, Ca^{2+} and Ni^{2+}, respectively. This resulted in a cumulative volume of 2968, 436, and 254 mL, respectively, for Mg^{2+}, Ca^{2+}, and Ni^{2+} (Figure 5.6 (B – D)). The biohydrogen yields for these metals were 263.7, 245.2 and 221.3 mL H_2/g TVS, respectively. These results were 23%, 17%, and 9% higher than the values obtained for Mg^{2+}, Ca^{2+}, and Ni^{2+}, respectively, in suspended cultures. These metals are essential for biohydrogen-producing pathways as well. For example, Mg^{2+} is used by glycolytic enzymes such as enolase and phosphorylase in metabolic pathways of various biohydrogen-producing microorganisms (Ding et al., 2004; Lee et al., 2001; Srikanth and Venkata Mohan, 2012; Wang and Wan, 2008; Zhang et al., 2005). Ca^{2+} maximizes spore-germination in *Bacillus* and *Clostridium* species during their exponential growth-phase (Garcin et al., 1999). Ni^{2+} affects the active structures of [Ni-Fe]-hydrogenase enzymes

(Karadag and Puhakka, 2008). Despite the enhancement ability of these divalent cations, it has been stated in several biohydrogen studies that high concentrations may results in the inhibition of biohydrogen-producing pathways (Singh and Wahid, 2014; Zhu et al., 2007). This phenomenon was observed in biohydrogen experiments of Ca^{2+} and Ni^{2+} ions whereby an optimum production was obtained at concentrations less than 1000 mg/L (100 and 500 mg/L) as shown in Figures 5.5 (C) and (D), respectively. It has also been proposed that this might be due to the coagulation effect which changes the charge distribution on the surface of bacterial cells (Zhu et al., 2007). The beads were also analyzed using a scanning electron microscopy (Figure 5.7) to assess their morphological changes during biohydrogen production i.e. before (A, C, E, G) and after (B, D, F, H) the process. The inner surface of used beads (B, D, F, H) was more porous due to utilization of nutrients by the entrapped bacteria. Therefore, the results obtained in this study showed that a dark fermentation process using metal ions and immobilized microorganisms is a promising approach that could be used to enhance the biohydrogen yields.


A
0 mg/L
50 mg/L
100 mg/L
300 mg/L
500 mg/L
1000 mg/L
H2 content (%)
Time (h)
0
5
10
15
20
25
30
35
40
45
50
0
20
40
60
80


B
0 mg/L
50 mg/L
100 mg/L
300 mg/L
500 mg/L
1000 mg/L
H2 content (%)
Time (h)
0
5
10
15
20
25
30
35
0
20
40
60
80

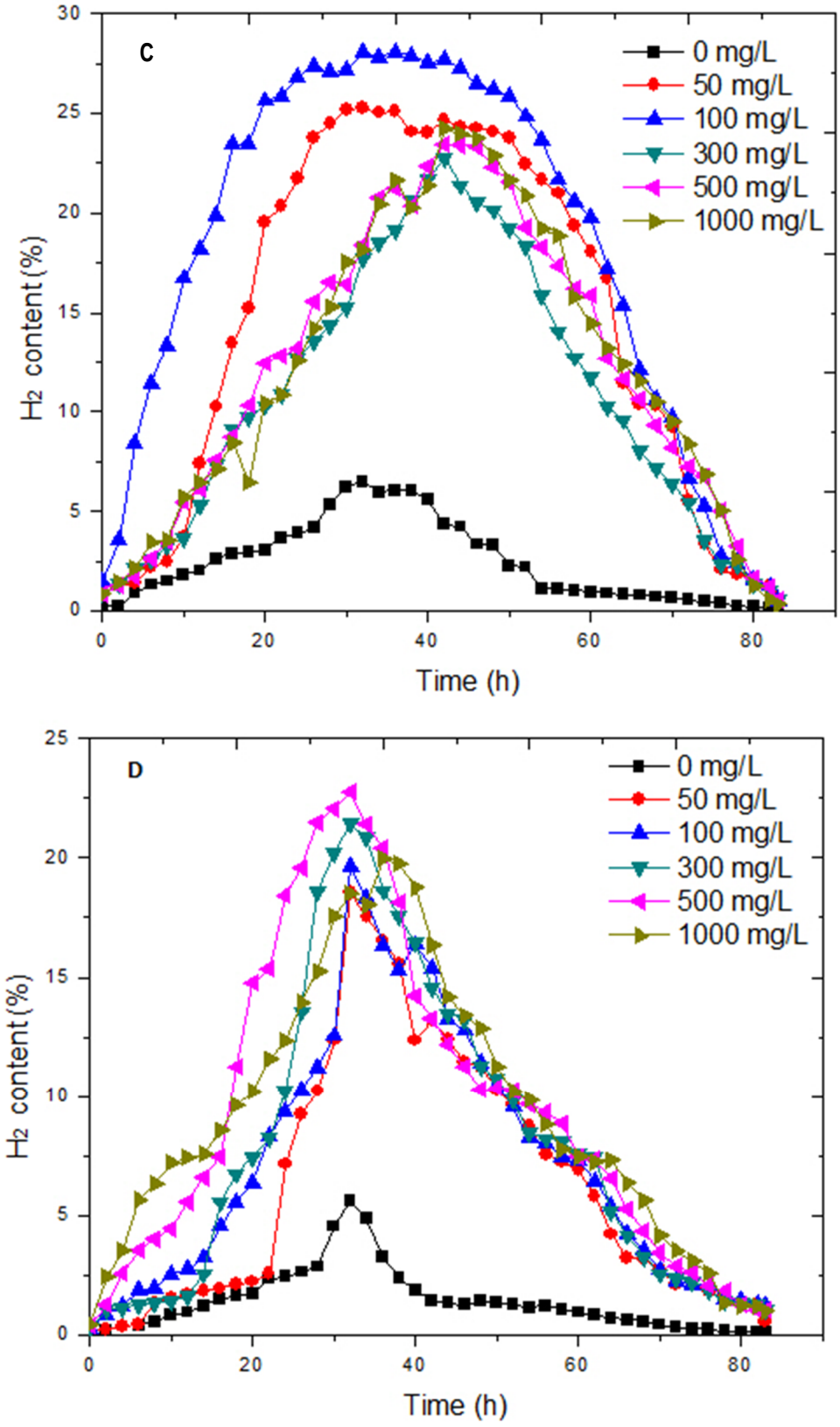
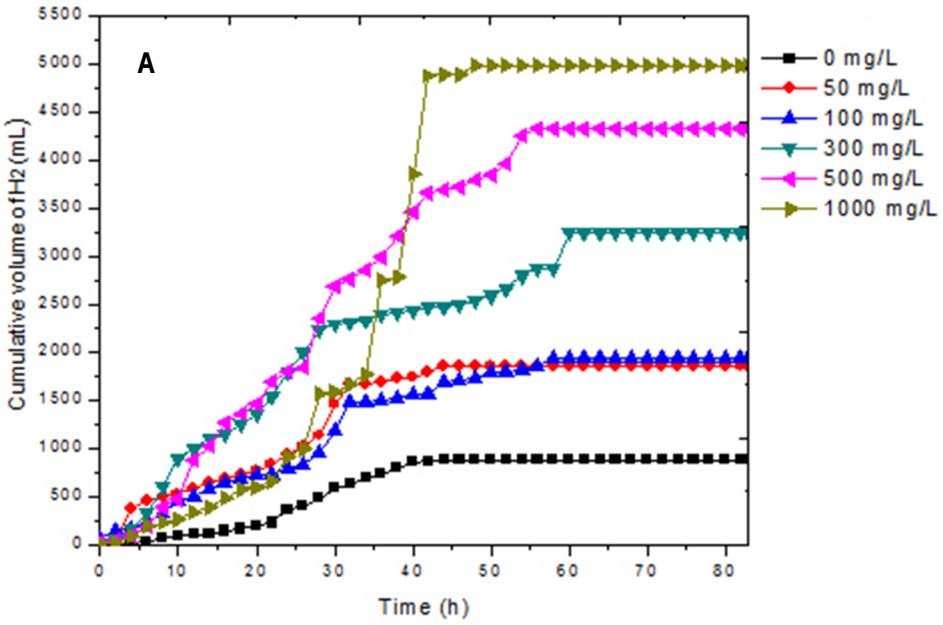
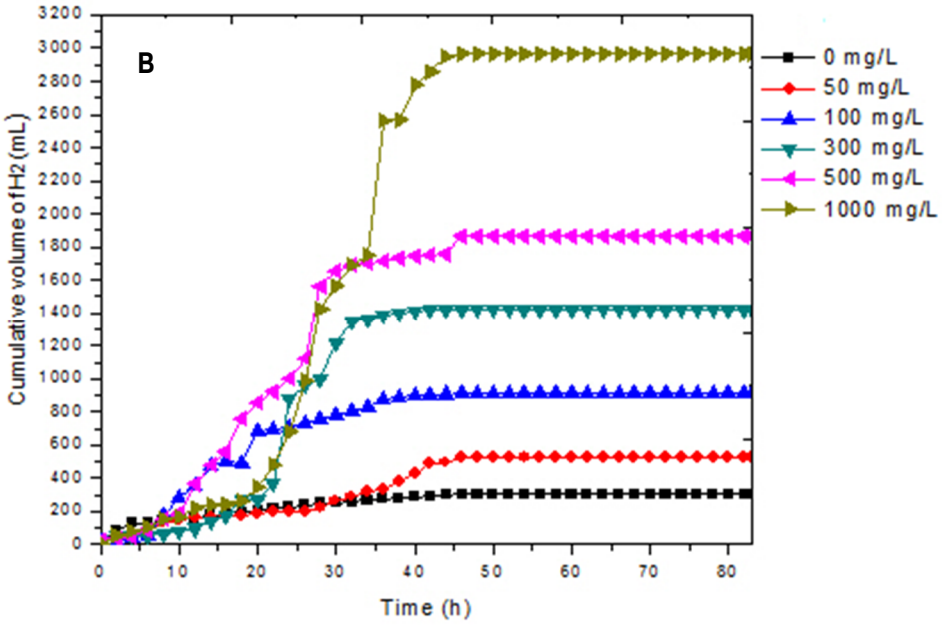


Figure 5.5: Effect of concentration (0-1000 mg/L) of Fe^{2+} (A), Mg^{2+} (B), Ca^{2+} (C) and Ni^{2+} (D) ion on biohydrogen production using immobilized bacteria.


A
Cumulative volume of H2 (mL)
Time (h)
0 mg/L
50 mg/L
100 mg/L
300 mg/L
500 mg/L
1000 mg/L


B
Cumulative volume of H2 (mL)
Time (h)
0 mg/L
50 mg/L
100 mg/L
300 mg/L
500 mg/L
1000 mg/L

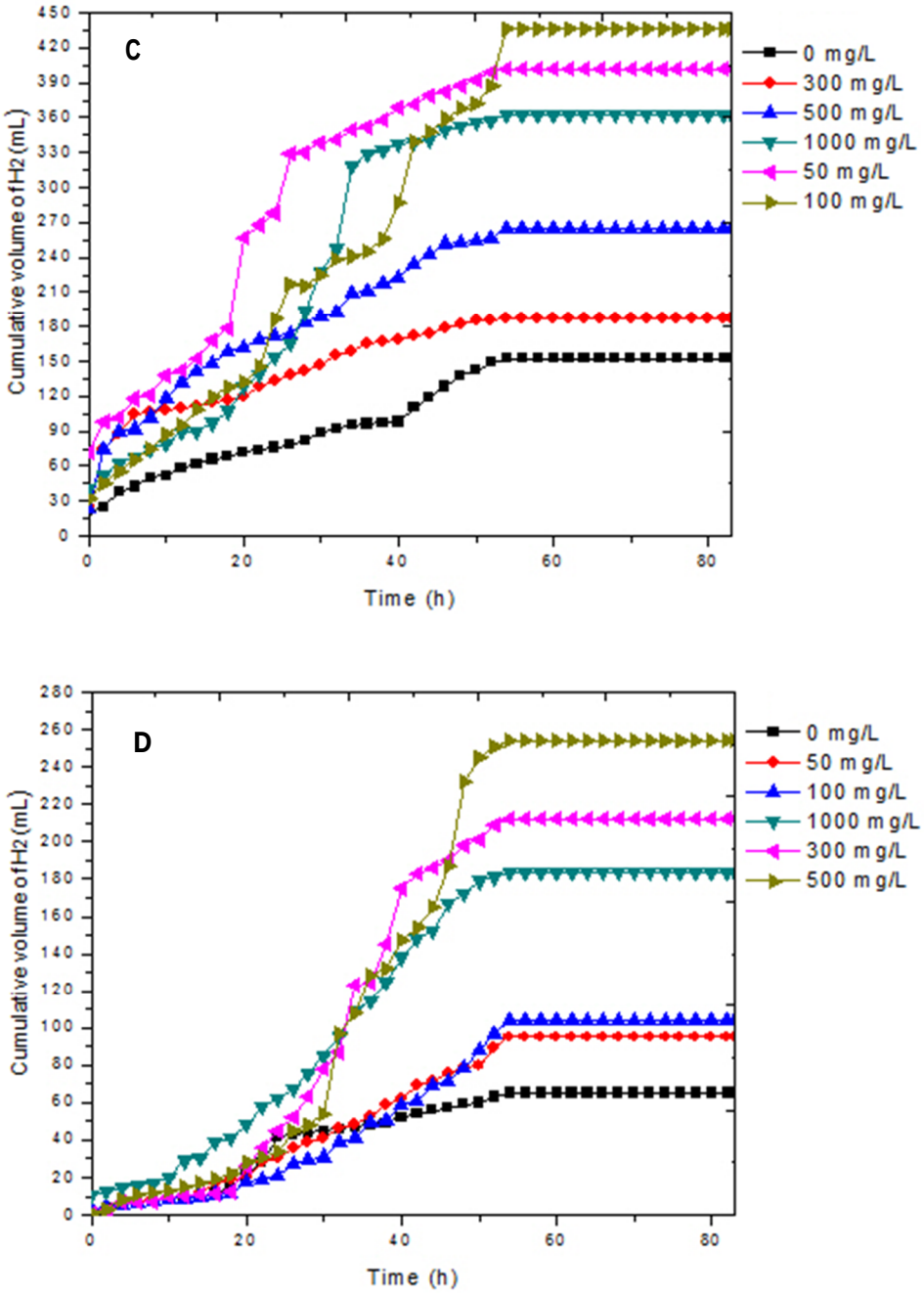
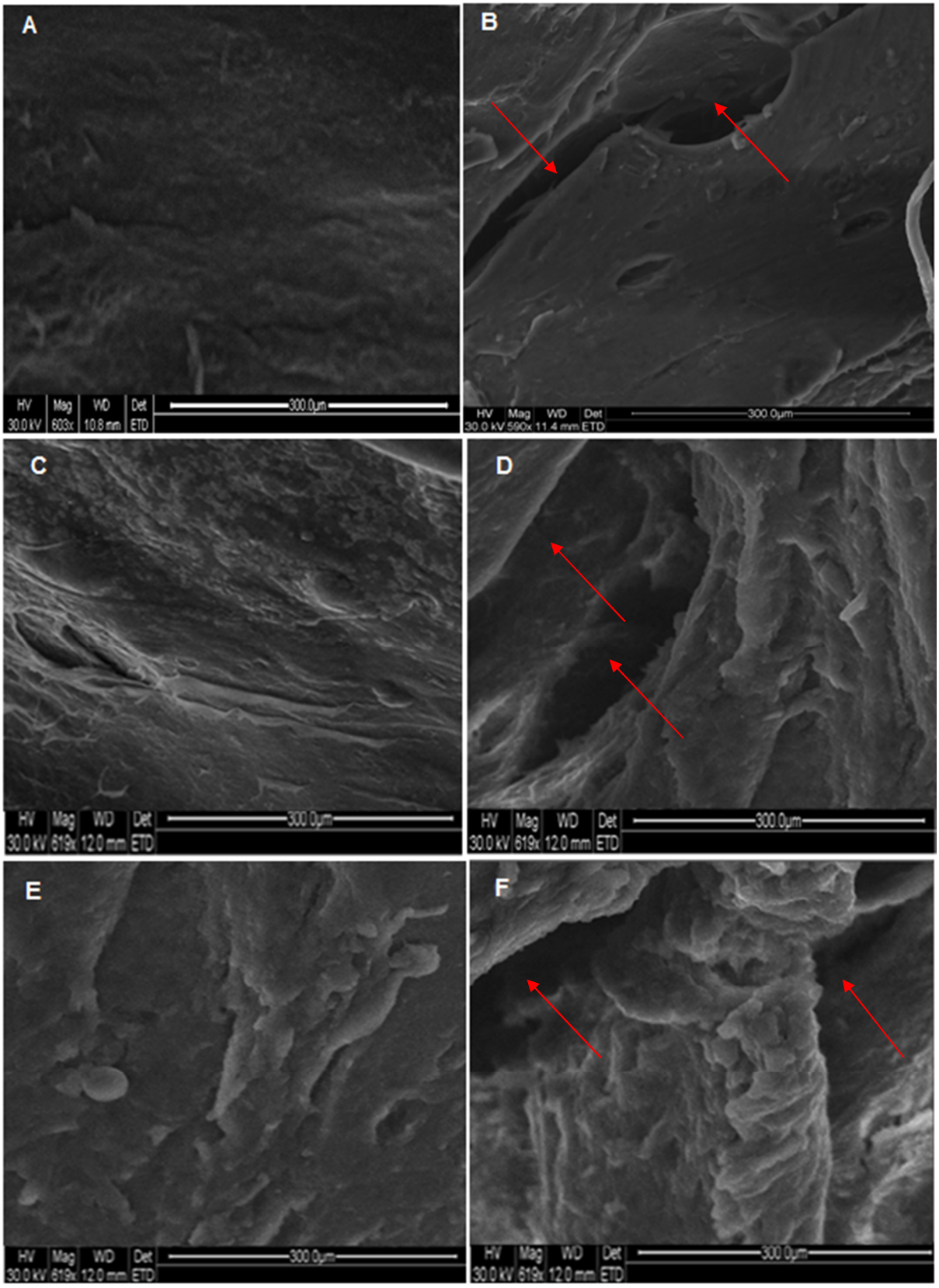


Figure 5.6: Effect of concentration (0-1000 mg/l) of Fe^{2+} (A), Mg^{2+} (B), Ca^{2+} (C) and Ni^{2+} (D) ion on cumulative biohydrogen production using immobilized bacteria.


A
HV 30.0 kV
Mag 603x
WD 10.8 mm
Det ETD
300.0µm
B
HV 30.0 kV
Mag 590x
WD 11.4 mm
Det ETD
300.0µm
C
HV 30.0 kV
Mag 619x
WD 12.0 mm
Det ETD
300.0µm
D
HV 30.0 kV
Mag 619x
WD 12.0 mm
Det ETD
300.0µm
E
HV 30.0 kV
Mag 619x
WD 12.0 mm
Det ETD
300.0µm
F
HV 30.0 kV
Mag 619x
WD 12.0 mm
Det ETD
300.0µm

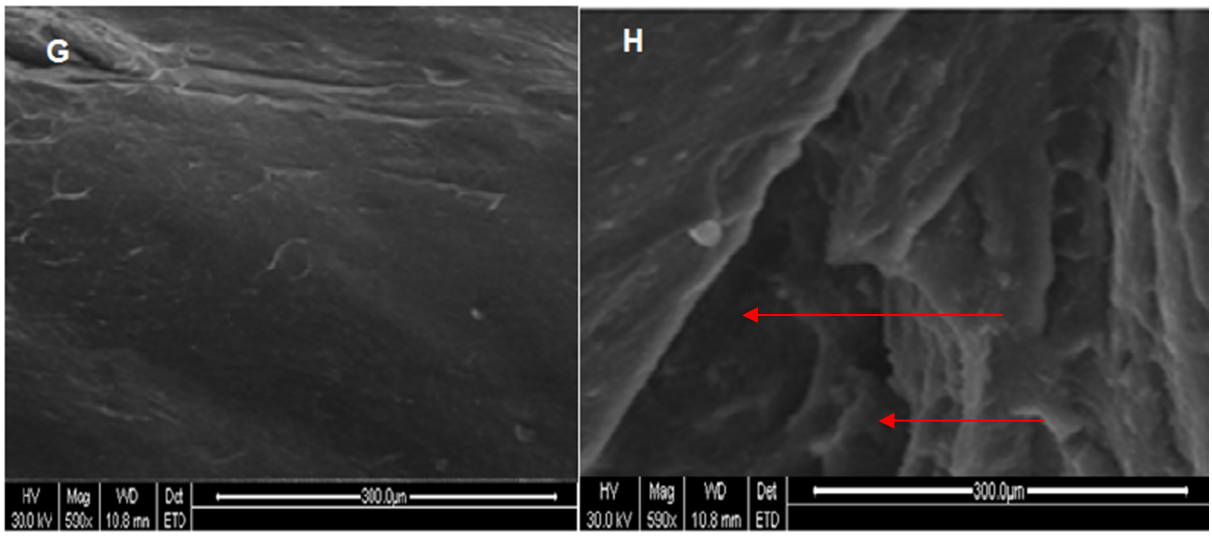


Figure 5.7: SEM images of calcium alginate beads taken before and after biohydrogen production. A – B: represent the beads used at Fe^{2+} concentration (1000 mg/L) before (A) and after (B) biohydrogen production. C – D: represent the beads used at Mg^{2+} concentration (1000 mg/L) before (C) and after (D) biohydrogen production. E – F: represent the beads used at Ca^{2+} concentration (100 mg/L) before (E) and after (F) biohydrogen production. G – H: represent the beads used at Ni^{2+} concentration (500 mg/L) before (G) and after (H) biohydrogen production. The pores on alginate beads were created by diffusion of nutrients, as indicated by the arrows.

5.3.3 Substrate degradation and pH change after the fermentation experiments

Potato waste served as a carbon source in this study and its utilization by the biohydrogen-producing bacteria was analyzed at the end of each batch test in the form of chemical oxygen demand (COD) as shown in Table 5.2. High substrate degradation was observed in experiments using Fe^{2+} and Mg^{2+} ions along with immobilized bacteria. In these batch tests, a COD removal efficiency of 52.30 and 49.89% was obtained for Fe^{2+} and Mg^{2+}, respectively, at concentrations of 1000 mg/L. These values were 4 and 7% higher than those of suspended cells (Table 5.2). A plausible contribution to high substrate conversion may be due to the ability of immobilized cells to withstand the soluble metabolites such as volatile fatty acids

and alcohols which rapidly change the buffering capacity of the medium during the solventogenesis process (Kourkoutas et al., 2004). The reaction is triggered by the accumulation of these intermediates and thus terminates biohydrogen production (Kourkoutas et al., 2004). The metal ions also play a crucial role in substrate degradation. For instance, Fe^{2+} helps in the release of electrons during microbial conversion of substrate to molecular hydrogen, whereas Mg^{2+} activates the molecules to enter the metabolic pathways during the acidogenic process (Xiao et al., 2013). This was also confirmed in other biohydrogen production studies. Karadag and Puhakka (2008) reported a substrate degradation of more than 99% in a dark fermentation process using Fe^{2+}. Srikanth and Venkata Mohan (2012) reported a maximum COD removal of 76.46 and 73.75%, respectively, for Fe^{2+} and Mg^{2+} at optimum concentration of 100 mg/L. Immobilized tests using Ca^{2+} and Ni^{2+} ions attained a COD removal efficiency of 37.65 and 37.25%, indicating a 13 and 2% increase in its degradation compared to suspended cultures (Table 5.2).

pH is one of the most crucial parameters in microbial hydrogen production because it affects the hydrogenase activity, proton gradient and substrate hydrolysis (Das and Veziroglu, 2001; Lin and Lay, 2005). This parameter was also monitored at each batch test. A slightly higher pH (4.56 compared to 3.63) was observed in the experiments using immobilized cells (Table 5.2). This could be attributed to the microbes being entrapped within the porous matrix, implying that they were not in direct contact with the inhibitory soluble intermediates produced during the acidogenic-solventogenic transition as highlighted earlier (Show et al., 2012). In a study conducted by Penniston and Gueguim Kana (2016), cell immobilization stabilized the buffering capacity of fermentation medium (pH was maintained at 4.5 for 10.5 h) and therefore extended biohydrogen production (Penniston and Gueguim Kana, 2016). In addition, the authors observed complete glucose degradation at peak production which suggested that the immobilized cells used all the substrate for their metabolic activities and

thereby producing hydrogen (Garcin et al., 1999). It is important to regulate pH during biohydrogen production because its variation may affect the uptake of nutrients, enzymatic reactions and DNA alteration (Penniston and Gueguim Kana, 2016). Incorporating sensors and actuators in biohydrogen processes will minimize the growth of biohydrogen-inhibiting microorganisms by maintaining pH medium that supports biohydrogen-producing reactions and thus improve its yield. However, the cost analysis will need to be performed at scale-up level.

Table 5.2: Effect of metal ions on COD removal and final pH during biohydrogen production experiments.

Metal ion	Suspended cells			Immobilized cells	
	Concentration (mg/L)	pH	COD removal (%)	pH	COD removal (%)
Fe^{2+}	0	3.78	36.57	4.56	46.36
	50	3.54	38.75	4.25	48.02
	100	4.56	40.02	4.81	48.56
	300	3.25	40.23	3.88	48.83
	500	3.07	48.56	3.75	49.68
	1000	3.36	50.23	3.86	52.30
Mg^{2+}	0	3.05	37.89	3.87	42.36
	50	3.21	38.89	4.02	43.26
	100	3.84	39.74	4.12	45.32
	300	3.76	40.56	4.16	46.38
	500	3.61	41.2	4.08	47.78
	1000	3.42	46.56	3.98	49.89
Ca^{2+}	0	3.41	30.25	3.99	31.75
	50	3.31	31.26	3.68	36.16
	100	3.21	33.25	3.78	37.65
	300	3.54	34.87	3.87	32.08
	500	3.05	35.61	3.69	31.87
	1000	3.41	36.25	3.74	30.15
Ni^{2+}	0	3.11	30.25	3.75	30.23
	50	3.01	31.05	3.56	30.81
	100	3.08	31.2	3.23	31.02
	300	3.14	34.12	3.41	32.02
	500	3.18	36.5	3.42	37.25
	1000	3.23	36.74	3.63	33.12

5.3.4 Biochemistry of metal ions on the metabolism of acidogenic bacteria

The functional role of these metals on the metabolism of biohydrogen-producing (acidogenic) microorganisms was studied in detail and is depicted in Figure 5.8. High concentrations of Fe^{2+} enhance fermentative biohydrogen production due to its metabolic roles on biohydrogen-producing hydrogenase enzymes and ferredoxin as highlighted earlier (Srikanth

and Venkata Mohan, 2012). Fe^{2+} acts as a mediator for intracellular electron transfer either independently or as a prosthetic group (Duran-Padilla et al., 2014). Fe^{2+} also helps the hydrogenase enzymes to reduce the loss of electrons during dark fermentation process which in turn enhances its production (Duran-Padilla et al., 2014). Meanwhile, Ni^{2+} affects the active site of biohydrogen-producing [Ni-Fe]-, and [Ni-Fe-Se]-hydrogenase enzymes which accelerate the production of biohydrogen and maintain an appropriate balance between electron donors and acceptors. However, it has been reported that [Ni-Fe-Se]-hydrogenases are used by biohydrogen-consuming methanogenic archaea and are responsible for the uptake of hydrogen (Garcin et al., 1999). Mg^{2+} plays a crucial role in substrate utilization especially for activation of substrate molecules during dark fermentation process. High concentrations of Mg^{2+} enable optimum levels of protons and electrons to reach hydrogenase enzymes and therefore improve its production. Mg^{2+} ions attaches to the adenosine triphosphate (ATP) molecule to form Mg-ATP, which reacts with glucose to form glucose-6-phosphate that enters the metabolic pathway as shown in Figure 5.8 (Moncrief and Maguire, 1999). Furthermore, Mg^{2+} is used by many electron carrier molecules such as cytochromes (Cyt C, Cyt a, a3) and protein complexes in various biological processes (Moncrief and Maguire, 1999). Ca^{2+} is involved in the growth of *Clostridium* species and stimulates the formation of endospores (Aran, 2001). It has been shown in some studies that Ca^{2+} ions assist in biofilm formation in certain bacterial species (Moncrief and Maguire, 1999). The functional role of other divalent ions such as Zn^{2+} and Mn^{2+} is also discussed in literature. Zn^{2+} is an important micronutrient that participates in the physiological processes of hydrogenase enzymes during acidogenic biohydrogen production process (Lin and Shei, 2008). Mn^{2+} possesses similar functions to Mg^{2+}; it is used by various endospore-forming species in their metabolic processes and accelerates their growth (Srikanth and Venkata Mohan, 2012). During dark fermentation, the H^+ is reduced to H_2 through a series of biochemical pathways i.e. H^+ is first

released from NADH dehydrogenase, membrane bound protein complexes facilitate the transfer of electrons, and is finally reduced to H_2 by [Fe-Fe]-, and [Fe-Ni]-hydrogenase enzymes as shown in Figure 5.8.

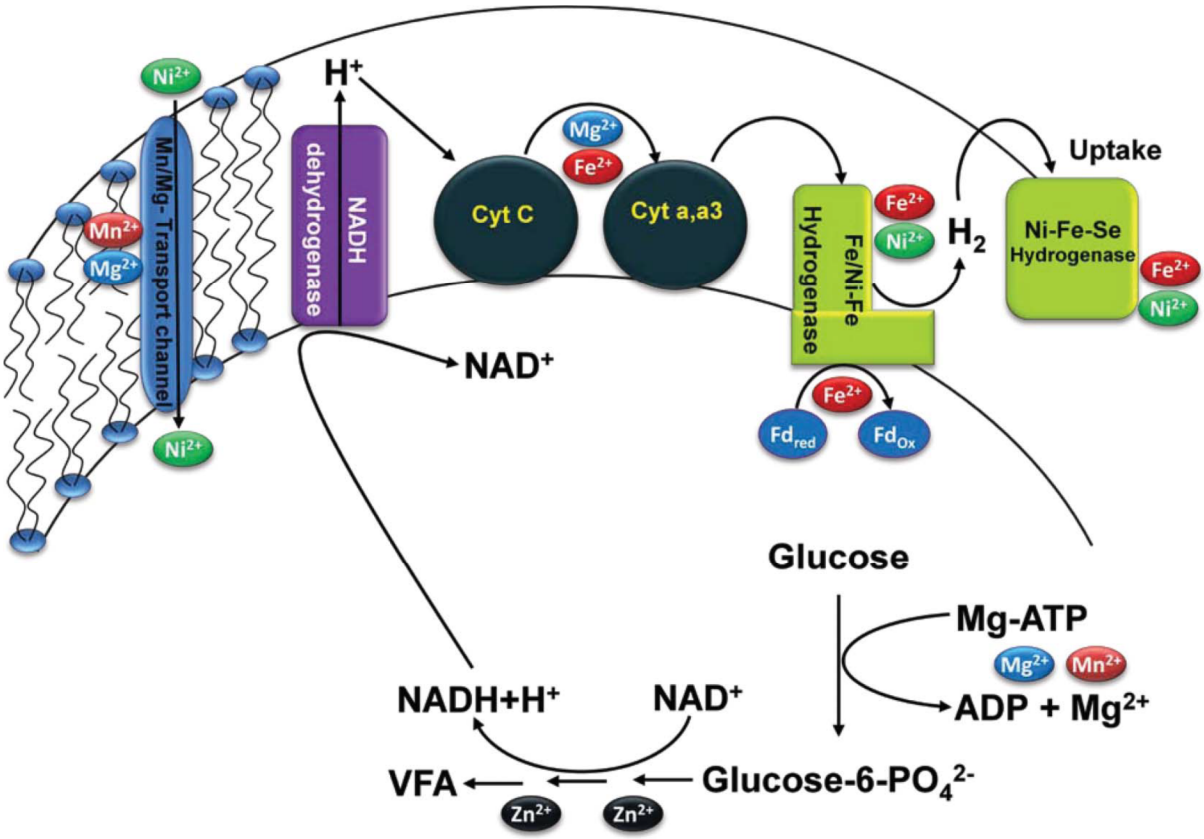


Figure 5.8: Schematic representation of the functional role of metal ions in the metabolism of biohydrogen-producing bacteria (Srikanth and Venkata Mohan (2012).

5.4 Summary

In this chapter, results of the evaluation of the effect of metal ions (Fe^{2+}, Ca^{2+}, Mg^{2+} and Ni^{2+}) on dark fermentative biohydrogen production using suspended and immobilized cells of anaerobic mixed bacteria are presented. A maximum biohydrogen fraction of 45.21%, corresponding to a yield of 292.8 mL H_2/g TVS was obtained in batch fermentation experiment using Fe^{2+} (1000 mg/L) and immobilized cells as the inoculum. The yield was 1.3 times higher than that of suspended cultures. In addition, a COD removal efficiency of 52.30% was also obtained during the experiment. The utilization of metal ions along with immobilized bacteria proved to be effective for enhancing biohydrogen production via dark fermentation and could be instrumental in overcoming low yield which hinders the commercialization of the process. These encouraging results have been sent to a reputable journal (Chemical Engineering Communications) for possible publication and the manuscript is currently under review (see Appendix A for a copy).

References

Alshiyab, H., Kalil, M.S., Hamid, A.A., and Yusof, W.WM. (2008). Effect of salts addition on hydrogen production by *C. acetobutylicum*. Pakistan Journal of Biological Sciences 11, 2193-2200.

Aran, N. (2001). The effect of calcium and sodium lactates on growth from spores of *Bacillus cereus* and *Clostridium perfringens* in a 'sous-vide' beef goulash under temperature abuse. International Journal of Food Microbiology 61, 117-123.

Argun, H., Kargi, F., and Kapdan, I.K. (2008). Light fermentation of dark fermentation effluent for bio-hydrogen production by different *Rhodobacter* species at different initial volatile fatty acid (VFA) concentration. International Journal of Hydrogen Energy 33, 7405-7412.

Bao, M.D., Su, H.J., and Tan, T.W. (2013). Dark fermentative bio-hydrogen production: Effects of substrate pre-treatment and addition of metal ions or L-cysteine. Fuel 112, 38-44.

Boni, M.R., Sbaffoni, S., and Tuccinardi, L. (2014). The influence of iron concentration on biohydrogen production from organic waste via anaerobic fermentation. Environmental Technology 32, 3000-3010.

Chang, F.Y., and Lin, C.Y. (2006). Calcium effect of fermentative hydrogen production in an anaerobic up-flow sludge blanket system. Water Science and Technology 54, 105-119.

Chong, M.L., Rahman, A.N., Yee, P.L., Aziz, A.S., Rahim, A.R., and Shirai, Y. (2009). Effects of pH, glucose and iron sulfate concentration on the yield of biohydrogen by *Clostridium butyricum* EB6. International Journal of Hydrogen Energy 34, 8859-8865.

Das, D., and Veziroglu, T.N. (2001). Hydrogen production by biological processes: a survey of literature. International Journal of Hydrogen Energy 26, 13-28.

Ding, J., Ren, N.Q., Liu, M., and Ding, L. (2004). Effect of Fe and Fe^{2+} on hydrogen production capacity with mixed culture. Environmental Science and Technology 25, 48–53.

Duran-Padilla, V.R., Davila-Vazquez, G., Chávez-Vela, N.A., Tinoco-Valencia, J.R., and Jáuregui-Rincón, J. (2014). Iron effect on the fermentative metabolism of *Clostridium acetobutylicum* ATCC 824 using cheese whey as substrate. Biofuel Research Journal 4, 129-133.

Eroglu, E., Gunduz, U., Yucel, M., and Eroglu, I. (2009). Photosynthetic bacterial growth and productivity under continuous illumination or diurnal cycles with olive mill wastewater as feedstock. International Journal of Hydrogen Energy 35, 5293-5300.

Garcin, E., Vernede, X., Hatchikian, E.C., Volbeda, A., and Fontecilla-Camps, J.C. (1999). The crystal structure of a reduced [NiFeSe] hydrogenase provides an image of the activated catalytic center. Structure 7, 557-566.

Karadag, D., and Puhakka, J.A. (2008). Enhancement of anaerobic hydrogen production by iron and nickel. International Journal of Hydrogen Energy 35, 8554-8560.

Kourkoutas, Y., Bekatorou, A., Banat, I.M., and Koutinas, A.A. (2004). Immobilization technologies and support materials suitable in alcohol beverages production: a review. Food Microbiology 21, 377-397.

Lee, D.Y., Li, Y.Y., Oh, Y.K., Kim, M.S., and Noike, T. (2009). Effect of iron concentration on continuous H_2 production using membrane bioreactor. International Journal of Hydrogen Energy 34, 1244-1252.

Lee, Y.J., Miyahara, T., and Noike, T. (2001). Effect of iron concentration on hydrogen fermentation. Bioresource Technology 80, 227–231.

Lin, C., and Lay, C. (2005). A nutrient formulation for fermentative hydrogen production using anaerobic sewage sludge. International Journal of Hydrogen Energy 30, 285-292.

Lin, C.N., Wu, S.Y., and Chang, J.S. (2006). Fermentative hydrogen production with a draft tube fluidized bed reactor containing silicone-gel-immobilized anaerobic sludge. International Journal of Hydrogen Energy 31, 2200-2210.

Lin, C.Y., and Shei, C.H. (2008). Heavy metal effects on fermentative hydrogen production using natural mixed microflora. International Journal of Hydrogen Energy 33, 587-593.

Liu, G., and Shen, J. (2004). Effects of culture and medium conditions on hydrogen production from starch using anaerobic bacteria. Journal of Biosciences and Bioengineering 98, 251–256.

Moncrief, M.B.C., and Maguire, M.E. (1999). Magnesium Transport in Prokaryotes. JBIC Journal of Biological Inorganic Chemistry 4, 523–527.

Paul, J.S., Quraishi, A., Thakur, V., and Jadhar, S.K. (2014). Effect of ferrous and nitrate ions on biological hydrogen production from dairy effluent with anaerobic waste water treatment process. Asian Journal of Biological Sciences 7, 1-7.

Penniston, J., and Gueguim Kana, E.B. (2016). Impact of Culture pH Regulation on Biohydrogen Production using Suspended and Immobilized Microbial Cells. Preprints, 1-21.

Show, K.Y., Lee, D.J., Tay, J.H., Lin, C.Y., and Chang, J.S. (2012). Biohydrogen production: Current perspectives and the way forward. International Journal of Hydrogen Energy 37, 15616-15631.

Singh, L., and Wahid, Z. (2014). Enhancement of hydrogen production from palm oil mill effluent via cell immobilisation technique. International Journal of Energy Research 39, 2215-222.

Srikanth, S., and Venkata Mohan, S. (2012). Regulatory function of divalent cations in controlling the acidogenic production process. RSC Advances 2, 6576-6589.

Wang, J.L., and Wan, W. (2008). Effect of Fe^{2+} concentration on fermentative hydrogen production by mixed cultures. International journal of Hydrogen Energy 33, 1215-1220.

Wongtanet, J., and Prapagdee, B. (2008). Effects of Inorganic Ions and Glucose on Hydrogen Production by Indigenous Microbes in Sewage Sludge. Environment Asia 2, 30-36.

Wu, K., Chang, J., and Chang, C. (2006). Biohydrogen production using suspended and immobilized mixed microflora. Journal of the Chinese Institute of Chemical Engineers 37, 545-550.

Xiao, L., Deng, Z., Fung, K.Y., and Ng, K.M. (2013). Biohydrogen generation from anaerobic digestion of food waste. International Journal of Hydrogen Energy 38, 1307-13913.

Yang, H., and Shen. J. (2006). Effect of ferrous iron concentration on anaerobic bio-hydrogen production from soluble starch. International Journal of Hydrogen Energy 31, 2137-2146.

Zhang, Y., Liu, G., and Shen, J. (2005). Hydrogen production in batch culture of mixed bacteria with sucrose under different iron concentrations. International Journal of Hydrogen Energy 30, 855–860.

Zheng, G., Kang, Z., and Qian, Y. (2009). Enhanced biohydrogen generation from organic wastewater containing $NH4^{+}$ by phototrophic bacteria *Rhodobacter sphaeroides* AR-3. Frontiers of Environmental Science and Engineering in China 3, 387-392.

Zheng, X.J., and Yu, H.Q. (2005). Inhibitory effects of butyrate on biological hydrogen production with mixed anaerobic cultures. Journal of Environmental Management 74, 65 – 70.

Zhu, H., Fang, H.P.P., Zhang, T., and Beaudette, L.A. (2007). Effect of ferrous ion on photo heterotrophic hydrogen production by *Rhodobacter sphaeroides*. International Journal of Hydrogen Energy 32, 4112-4118.

Chapter 6 – Effect of nitrogen gas sparging on dark fermentative biohydrogen production using suspended and immobilized cells of anaerobic mixed bacteria

The results obtained from the investigation of the effect of nitrogen gas sparging on dark fermentative biohydrogen production performance using suspended and immobilized cells of anaerobic mixed sludge are presented in this chapter. As far as could be ascertained, there are currently no studies in literature that evaluate the influence of nitrogen gas sparging on biohydrogen production using immobilized cells. Therefore, this work is the first open report on the study and it serves as a platform upon which subsequent research development could be built to provide in-depth understanding on the mechanism of nitrogen gas sparging on biohydrogen production performance using immobilized biocatalysts.

6.1 Introduction

Hydrogen partial pressure in the liquid phase has been identified as one of the most important parameters affecting the biohydrogen yield (Bundhoo and Mohee, 2016; Levin et al., 2004). During biohydrogen production, the hydrogen partial pressure in the liquid phase increases and causes the process to be thermodynamically inhibited because the reduction of ferredoxin (membrane-bound protein that facilitates the tranfer of electrons) is favoured resulting in the oxidation of hydrogen to protons (Equation 6.1), thereby decreasing its overall production (Chong et al., 2009). It has been shown in various studies that an increase in hydrogen partial pressure shifts the microbial activities towards biohydrogen-inhibiting reactions such as lactate, ethanol, acetone and butanol fermentation pathways (Bastidas-Oyanedel et al., 2012; Guo et al., 2010; Oh et al., 2009; Karlsson et al., 2008; Kim et al., 2006; Kraemer and Bagley, 2008; Levin et al., 2004; Mandal et al., 2006; Tanisho et al., 1998).

$$Fd_{ox} + H_2 \rightarrow F_{red} + 2H^+ \quad (6.1)$$

Therefore, maintaining a low partial pressure of hydrogen is significant because it favours the formation of hydrogen and permits microorganisms to metabolize the acetyl-CoA through the biohydrogen-producing pathways leading to acetate and adenosine triphosphate (ATP) production (Khanal et al., 2004). Several approaches have been employed to decrease the hydrogen partial pressure. These include vacuum stripping (Foglia et al., 2011), application of permeable membranes (Jung et al., 2011), and larger volume headspace (Oh et al., 2009). However, these strategies are expensive and will escalate the process costs (Ghimire et al., 2015). Gas sparging is one of the simplest method of reducing the partial pressure and is widely used in biological hydrogen production experiments to improve its yield (Bru et al., 2012; Kim et al., 2006; Mizuno et al., 2000; Nguyen et al., 2010; Pachapur et al., 2015). Nyugen et al. (2010) reported a 78% biohydrogen production increase when the reactor was continuously being purged with nitrogen gas. Minuzo et al. (2000) observed a 68% increase in biohydrogen production under nitrogen gas sparging. In another study, Kim et al. (2006) compared two biohydrogen production systems (sparged and non-sparged) and observed an increase in systems sparged with nitrogen and carbon dioxide gas. However, all these experimental studies were carried out using suspended cultures. This chapter was therefore conducted to investigate the effect of nitrogen gas sparging on biohydrogen production using both suspended and immobilized cells of anaerobic mixed sludge. To the best of our knowledge, studies evaluating the effect of nitrogen gas sparging using immobilized microbial cells are not yet documented in literature. The process performance was based on the key parameters such as biohydrogen yield, variation in pH, chemical oxygen demand removal efficiency, and volatile fatty acids production. These parameters have been used as indicators in literature for examining the biohydrogen production experiments using sparged microbial cells (Kim et al., 2006; Mizuno et al., 2000; Veeravalli et al., 2014).

6.2 Materials and methods

6.2.1 Substrate and inoculum preparation

The potato waste was prepared using the procedure outlined in section 3.2.1. The anaerobic mixed sludge was subjected to heat pretreatment as indicated in section 3.2.2. Further analysis was carried out in this chapter by determining the composition of potato waste and anaerobic sludge. Therefore, parameters such as biochemical oxygen demand (BOD), chemical oxygen demand (COD), volatile suspended solids (VSS), total Kjeldahl nitrogen (TKN), total solids (TS), and total volatile solids (TVS) were determined using the standard methods (APHA, 1998). pH was measured using a pH Meter Basic 20+ (Crison, South Africa). The characteristics of potato waste and anaerobic mixed sludge are presented in Tables 6.1 and 6.2, respectively.

Table 6.1: Characteristics of anaerobic mixed sludge.

Parameter (mg/L)	Value
COD	2004±1630
VSS	1012±428
TS	1314±1820
TVS	325±720
pH	6.2±0.2

Table 6.2: Characteristics of potato waste.

Parameter (mg/L)	Value
COD	2210±1852
BOD	1623±1520
TKN	198±121
TS	1123±1310
TVS	835±456
pH	6.8±0.2

6.2.2 Biohydrogen production experiments

Batch fermentative biohydrogen production experiments were conducted in 1 L modified Erlenmeyer flask reactors. The reactors were fed with 50 mL of liquid sludge consisting of biohydrogen-producing spore-forming bacteria (Figure 6.1 (A)) and 450 mL of synthetic medium consisting of the following (g/L): sucrose 10, NH_4HCO_3 2.0, NH_4Cl 0.5, $Na_2MoO_4.2H_2O$ 0.025, KH_2PO_4 0.25, $ZnCl_2$ 0.0115, $CuCl_2$ 0.0105, $MnSO_4$ 0.005 and $MnCl_2$ 0.015. Prior to the fermentation process, the reactors were sparged with nitrogen gas for 5 minutes and immediately sealed with silicone rubber stoppers to create conditions that are suitable for biohydrogen-producers. After 24 hour of operation, the reactors were routinely sparged with nitrogen gas at 1 hour intervals to reduce the hydrogen partial pressure in the liquid phase. According to Mizuno et al. (2000), this technique is highly effective against the build-up of hydrogen in the liquid phase and suppresses the activity of biohydrogen-consuming bacteria. The operating conditions were 5.56, 37.87 ^oC, and 82.58 hours for pH, temperature, and fermentation time, respectively. These variables were obtained from the parametric optimization study reported in chapter 4. The initial pH was adjusted with no further control. For the experiments involving the encapsulated cells, the sludge was first immobilized using the protocol in section 4.2.5.1. The batch reactors were inoculated with 130 g of alginate beads (Figure 6.1 (B)) and the abovementioned support medium. They were also sparged with nitrogen gas as stated above. The operating conditions were kept the same. A control experiment was conducted at similar operating conditions using suspended cells without further sparging. All experiments were conducted in duplicate for accuracy of data and reduction in experimental error. Experiments were conducted in a temperature-regulated stirring hot-plate at agitation speed of 100 rpm.

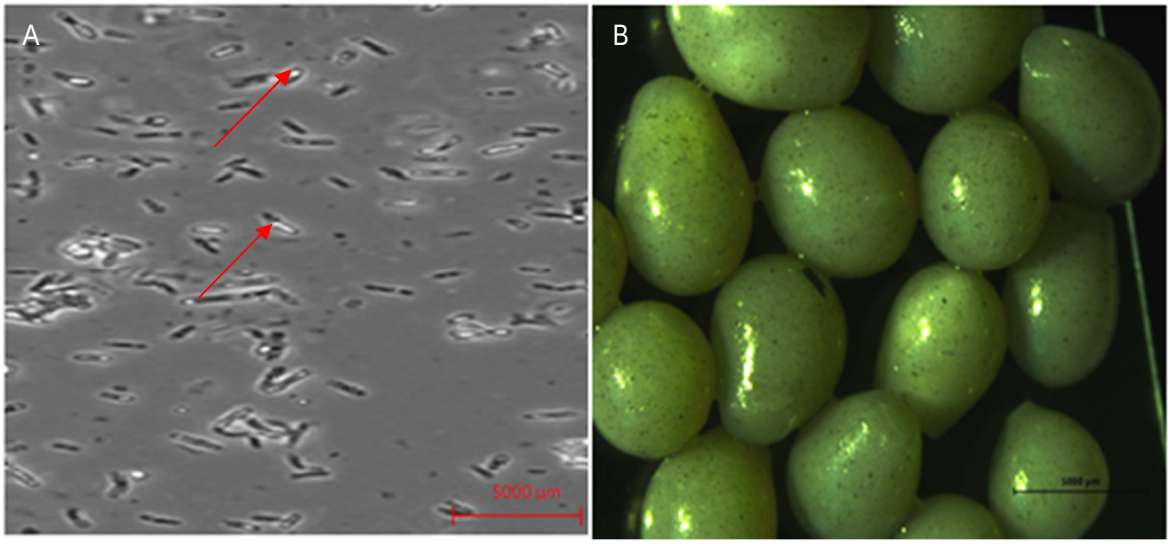


Figure 6.1: Morphology of biohydrogen-producing spore-forming bacteria in anaerobic mixed sludge (A) and bacteria immobilized in alginate beads (B). The spores are indicated with an arrow.

6.2.3 Analysis

In this study, parameters such as pH, biohydrogen fraction, cumulative biohydrogen, chemical oxygen demand (COD), volatile fatty acids (VFAs), and total volatile solids (TVS) were determined using the procedures in section 3.2.6. The morphology of spore-forming biohydrogen-producing bacteria was observed using an Olympus AX70 light microscope (Tokyo, Japan), whereas bacteria immobilized in alginate beads were examined using a Nikon SMZ745T stereomicroscope (Tokyo, Japan) as indicated in section 4.2.5.4.

6.3 Results and discussion

6.3.1 Effect of nitrogen gas sparging on biohydrogen production using suspended cells

Biohydrogen production commenced after a lag phase of 2 h and reached an optimum fraction of 40.01% at 42 h (Figure 6.2 (A)) and a cumulative volume of 2360 mL (Figure 6.2 (B)). There was a steady decrease in biohydrogen concentration at 44 – 82 h which may be due to depletion of nutrients and accumulation of metabolites such as volatile fatty acids and

alcohols (Srikanth and Venkata Mohan, 2012). The process attained a biohydrogen yield of 163.63 mL H_2/g TVS. Meanwhile, the control (non-sparged system) experiment produced an optimum biohydrogen fraction of 38.69% at 40 h (Figure 6.3 (A)) and a cumulative volume of 1230 mL (Figure 6.3 (B)). This process generated a biohydrogen yield of 117.81 mL/g TVS which was 28% lower than that of the sparged system. Based on these results, it can be concluded that nitrogen gas sparging was effective against the build-up of partial pressure which led to an enhanced biohydrogen production. This phenomenon was confirmed in studies evaluating the efficiency of nitrogen gas sparging on biohydrogen production yield. Veeravalli et al. (2014) maximized the biohydrogen yield by 46% in dark fermentation process using switchgrass liquor. In addition, nitrogen gas sparging was effective in enriching the predominant species such as *Clostridium* spp. (Veeravalli et al., 2014). Yerushalmi et al. (1985) reported that biohydrogen-inhibiting reactions of butanol and ethanol were favoured as hydrogen partial pressure increased and resulted in a 30% drop in biohydrogen yield. Beckers et al. (2012) studied the effect of partial pressure and observed a biohydrogen yield increase of 9.2% and 22.5% when the pressure was reduced from 1.18 to 1 bar, respectively. In another study, Logan et al. (2002) indicated that reducing the partial pressure in the reactor headspace led to a 43% increase in biohydrogen yield. Nitrogen gas sparging is beneficial in dark fermentation process because it inhibits the growth of biohydrogen-scavenging microorganisms and extends the acidogenic process (Bastidas-Oyanedel et al., 2012; Karlsson et al., 2008; Kim et al., 2006; Kraemer and Bagley, 2008; Tanisho et al., 1998). Furthermore, it improves the transfer of hydrogen from the liquid to the gas phase thus making it inaccessible to these organisms during the fermentation process (Mizuno et al., 2000; Nguyen et al., 2010; Pachapur et al., 2015). Bastidas-Oyanedel et al. (2012) studied the mechanism of gas sparging on biohydrogen related pathways of lactate hydrogenase, NADH hydrogenase and homoacetogenesis to fully understand its effects on biohydrogen-producing

reactions. The authors demonstrated that low partial pressure reduces the concentration of pyruvate, increases substrate oxidation and consequently prevents the synthesis of lactate. The inhibition of lactate favoured acidogenesis (Bastidas-Oyanedel et al., 2012). NADH hydrogenase is one of the key enzymes involved in biohydrogen production, it produces hydrogen (H_2) from the oxidation of NADH to NAD^+ as shown in Equation 6.2 (Bastidas-Oyanedel et al., 2012). It was revealed that the process is not thermodynamically feasible in non-sparged systems because the accumulation of pressure decreases the NAD^+/NADH ratio and thus reduces biohydrogen production (Bastidas-Oyanedel et al., 2012). In the case of homoacetogenic process, they observed that the process was inhibited at partial pressure lower than 0.02 bar (Bastidas-Oyanedel et al., 2012). Hence, these reports highlight the importance of reducing the partial pressure via gas sparging in order to enhance the performance of dark fermentative biohydrogen production.

$$NADH + H^+ \rightarrow H_2 + NAD^+ \quad (6.2)$$

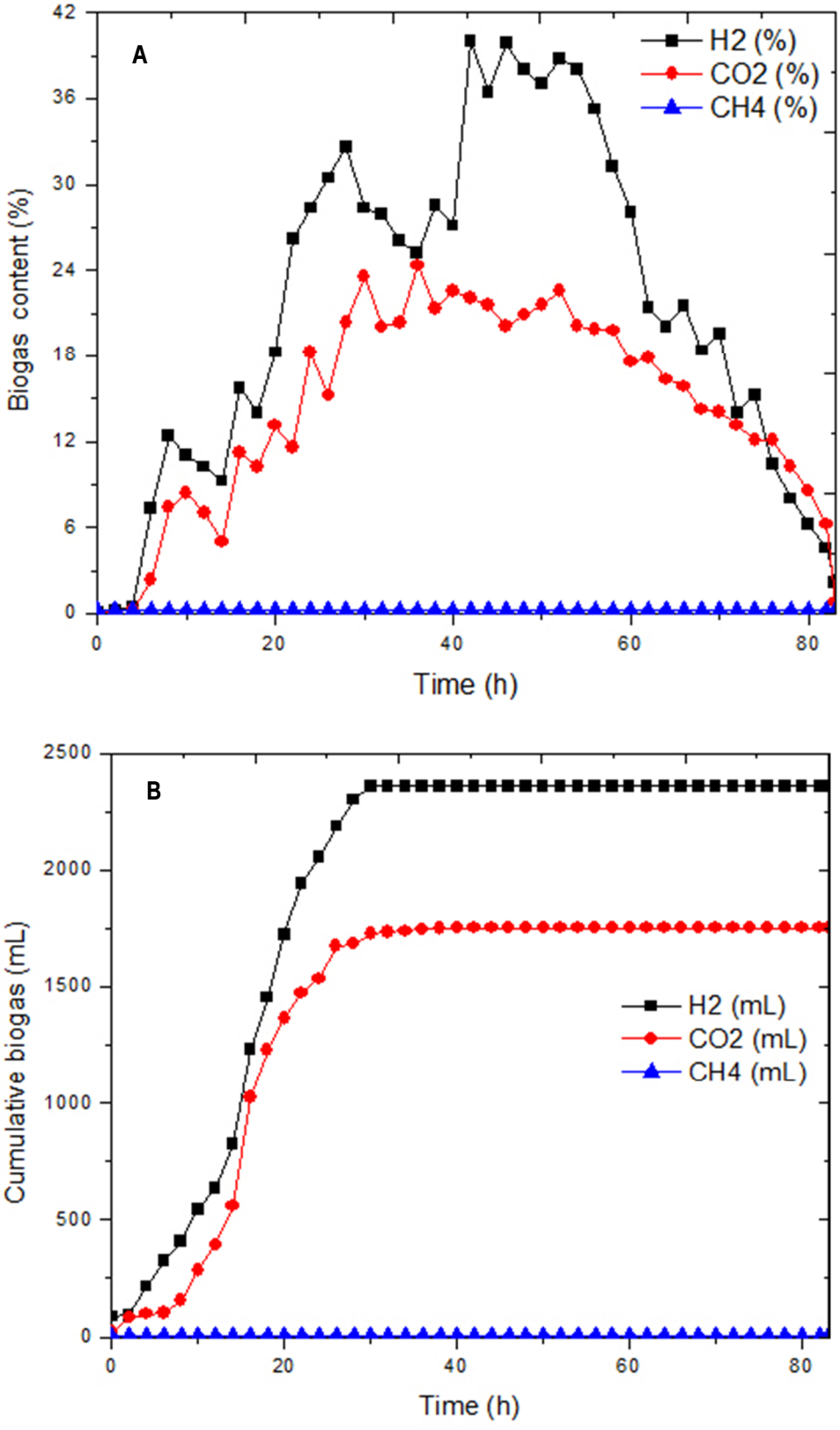


Figure 6.2: Biogas (hydrogen, carbon dioxide, and methane) produced during biohydrogen production using nitrogen gas sparged suspended cells (A) and the cumulative biogas (B).

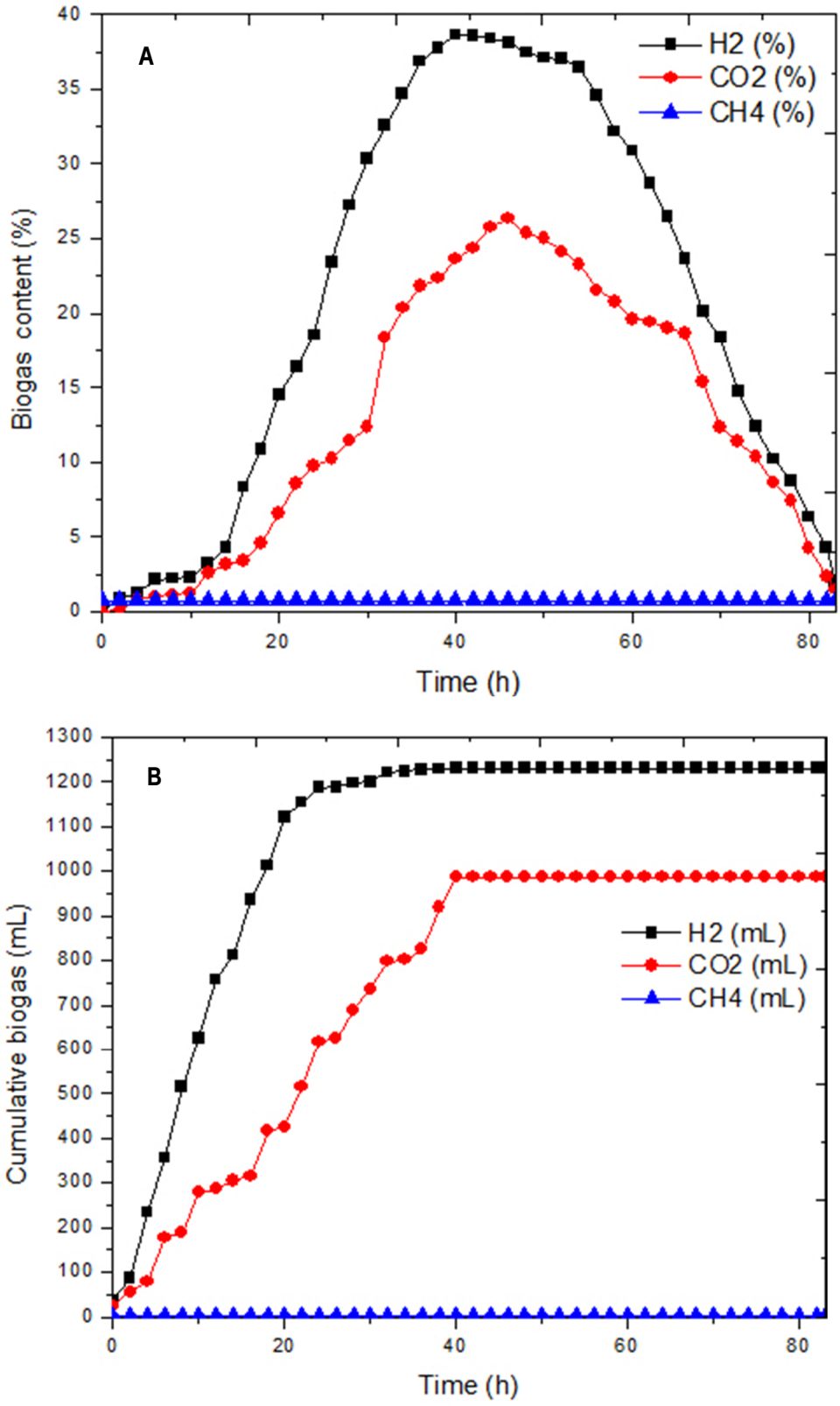


Figure 6.3: Biogas (hydrogen, carbon dioxide, and methane) produced during biohydrogen production using non-sparged suspended cells (A) and the cumulative biogas (B).

6.3.2 pH evolution in nitrogen gas sparged and non-sparged suspended cells

pH is considered to be one of the most important parameters governing biohydrogen production because it affects hydrogenase activity, metabolic pathways, and substrate hydrolysis (Fan et al., 2006; Van Ginkel et al., 2001). Hence, a comparative study was conducted to evaluate the change in pH in nitrogen gas sparged and non-sparged (control) suspended cell systems. pH shifted towards the acidophilic range as shown in Figure 6.4. The final pH of sparged and non-sparged (control) suspended cell system followed the usual anaerobic digestion trend i.e. it was below the acceptable value (pH 4) due to a switch in metabolic pathways from acidogenic to solventogenic processes (Venkata Mohan, 2009). The process of solventogenesis is induced by variability in intracellular pH and exhaustion of nutrients (Chandrasekhar et al., 2015). This leads to the formation of inhibitory by-products such as volatile fatty acids and alcohols which terminate biohydrogen-producing reactions (Yasin et al., 2013). Nicolaou et al. (2010) showed that these metabolites can disrupt the functional ability of cell membranes and might cause cell death. pH values ranging from 4−8 have been proposed in biohydrogen production studies (Nicolaou et al., 2010).

6.3.3 COD removal efficiency in nitrogen gas sparged and non-sparged suspended cells

The ability of suspended cultures to convert the substrate (potato waste) into biohydrogen was also examined by calculating the amount of chemical oxygen demand (COD) consumed during the fermentation process. A maximum COD removal efficiency of 47.5% and 42.5% was obtained in sparged and non-suspended system, respectively (Figure 6.5). A plausible contribution to high COD removal efficiency in sparged system is due to the fact that nitrogen gas sparging suppresses the activity of biohydrogen-consuming bacteria and therefore extends the metabolic activity of acidogenic bacteria as highlighted earlier (Mizuno et al., 2000; Nguyen et al., 2010; Pachapur et al., 2015). Potato also served as a suitable substrate to these organisms because it is rich in nutritional content (80-95% volatile solids

and 75-85% moisture) and is easily hydrolyzed by bacteria (Kumar et al., 2016; Xiao et al., 2013).

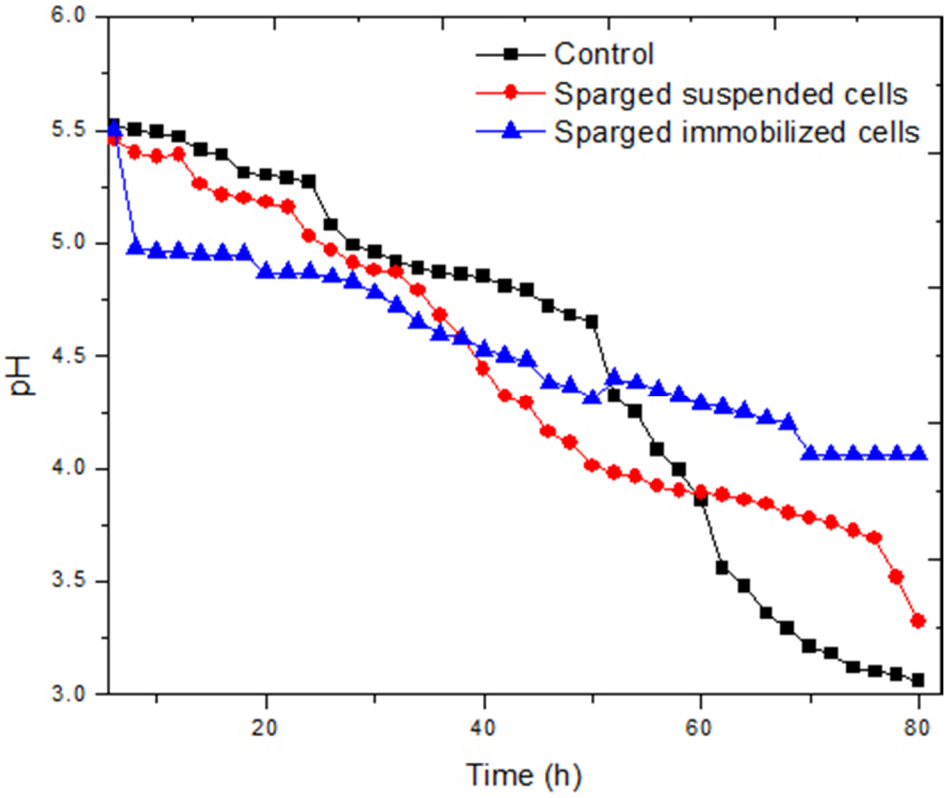


Figure 6.4: Variation of pH in dark fermentative biohydrogen production experiments.

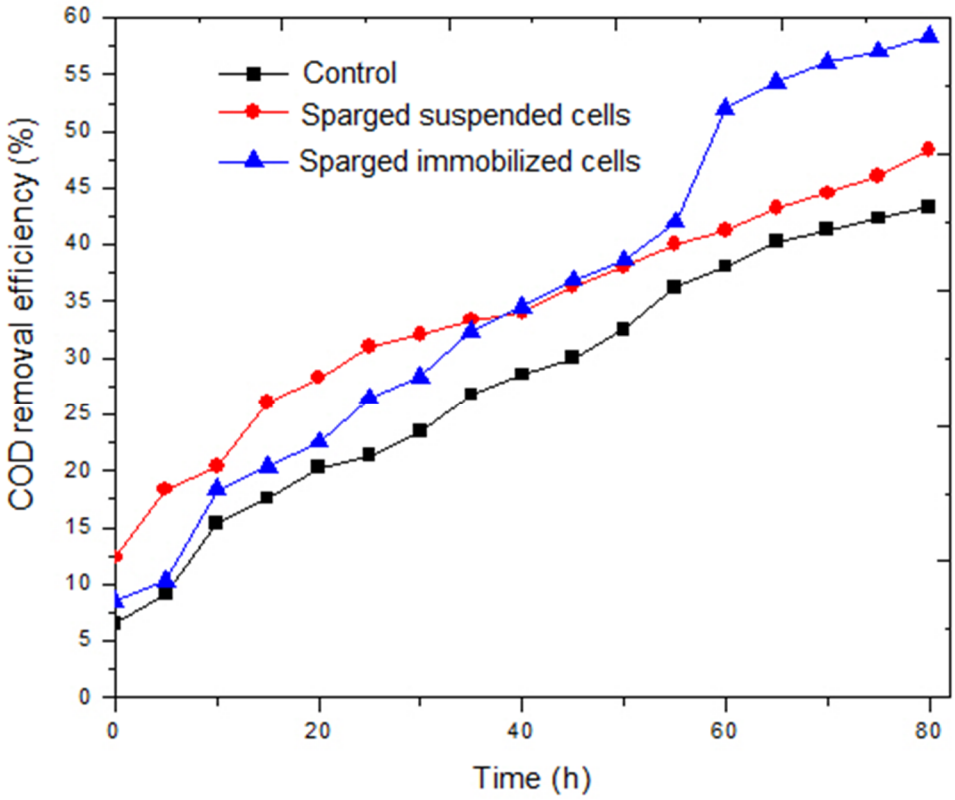


Figure 6.5: COD removal in dark fermentative biohydrogen production experiments.

6.3.4 Effect of nitrogen gas sparging on biohydrogen production using immobilized cells

A dark fermentation process was carried out to evaluate the performance of nitrogen gas sparging using immobilized bacteria. Figures 6.6 (A) and (B) show the biohydrogen fraction and the corresponding cumulative volume obtained using immobilized cells of anaerobic mixed sludge. The production of biohydrogen started after a lag phase of 3 h and increased exponentially to reach a maximum fraction of 56.98% at 66 h (Figure 6.6 (A)), and a cumulative volume of 4321 mL (Figure 6.6 (B)). This was followed by a sharp decline in biohydrogen fraction due to a switch in metabolic activities as mentioned earlier (Xiao et al., 2013). Nevertheless, the process produced a substantial yield of 294.83 mL H_2/g TVS which was 1.8 and 2.5 times higher than those obtained for the sparged and non-sparged (control) suspended cell systems, respectively. The high biohydrogen yield obtained in this experiment could be attributed to the synergistic effects of nitrogen gas sparging and cell immobilization on biohydrogen-producing bacteria which could result in improved homogeneity, low partial pressure, stable pH, shortened lag phase, high cell concentration, and high substrate conversion efficiency (Kumar et al., 2016; Ma et al., 2017). However, more studies needs to focus on parameters such as bead size, immobilizing matrix, bead morphology, and permeability in order to improve the overall biohydrogen production performance.

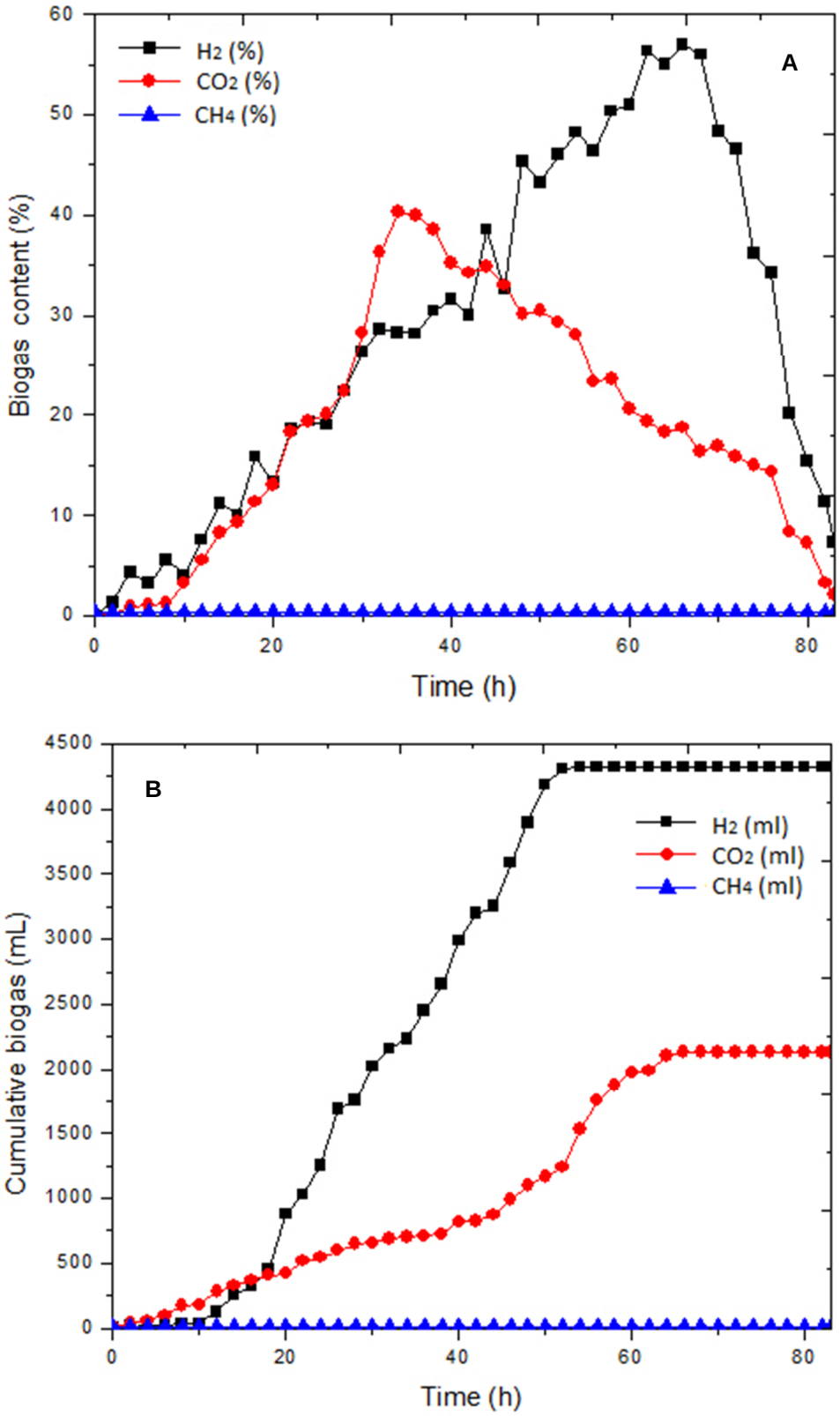


Figure 6.6: Biogas (hydrogen, carbon dioxide, and methane) produced during biohydrogen production using nitrogen gas sparged immobilized cells (A) and cumulative biogas (B).

6.3.5 pH evolution in nitrogen gas sparged immobilized cells

The system displayed a final pH of 4.02 as indicated in Figure 6.4. This implies that the sparged immobilized cells improved the buffering capacity of the medium compared to the suspended cultures. Several studies have shown that immobilized microorganisms are less sensitive to change in pH because the encapsulation barrier (immobilization matrix) protects them against the metal ions which reduce the buffering capacity of the medium (Kumar et al., 2016; Sekoai et al., 2017; Xiao et al., 2013). In similar studies, Penniston and Gueguim Kana (2016) observed that encapsulation of bacteria in sodium alginate increased the buffering capacity of the medium i.e. pH was maintained at 4.5 for more than 10 h. Keskin et al. (2002) achieved a five-fold biohydrogen production increase and sustained the pH at 4.5–5.0 in a fermentation system using ceramic beads as support material. Although there has not been any study in literature that examine the effect of nitrogen gas sparging on biohydrogen production using immobilized cells, the sparging could have also contributed to the inhibition of solventogenic reactions which rapidly reduces the medium pH due to the formation of acid metabolites (Keskin et al., 2002).

6.3.6 COD removal efficiency in nitrogen gas sparged immobilized cells

The immobilized system prolonged the exponential growth phase (2-66 h) of the acidogenic process resulting in high COD removal efficiency of 57.52% (Figure 6.5). These results coincide with literature. Penniston and Gueguim Kana (2016) observed complete glucose consumption at peak biohydrogen production which suggested that the immobilized cells used all the substrate for their metabolic activities to produce biohydrogen. Cell immobilization extends the biohydrogen-producing acidogenic process because the microorganisms are not in direct contact with the inhibitory fermentation metabolites such as toxic metals, volatile fatty acids, and alcohols which rapidly drift the pH of the medium during the acidogenic-solventogenic transition (Keskin et al., 2002; Ma et al., 2017).

6.3.7 Volatile fatty acids production in sparged suspended and immobilized cells

Volatile fatty acids (VFAs) analysis was conducted in this study to understand the effect of nitrogen gas sparging on their production. Therefore, these metabolites were evaluated in biohydrogen fermentation experiments of nitrogen gas sparged immobilized cells and nitrogen gas sparged suspended cells, respectively. The main VFAs were acetate, butyrate, and propionate. The technique of nitrogen gas sparging and cell immobilization enhanced the production of biohydrogen as indicated in this study and this led to a proportional increase in VFAs production due to the stoichiometric relationship of acetate and butyrate-fermentation reactions (see Equations 6.3 and 6.4), respectively. The process for nitrogen sparged immobilized cells accounted for 58.36%, 32.02%, and 16.36% of acetate, butyrate and propionate, respectively, during peak production phase (50 h) as shown in Figure 6.7 (A). The concentrations for the sparged suspended cell system (Figure 6.7 (B)) were 53.18%, 26.05%, and 18.56% for acetate, butyrate, and propionate, respectively. Members of predominant biohydrogen-producing taxa such as *Clostridium* sp. also produce VFAs along with biohydrogen during their exponential growth phase and switch to alcohol fermentation in the late growth phase (Mohanakrishna et al., 2010; Oh et al., 2009). These results suggest that the microbial consortia used in this study possessed clostridial characteristics as indicated by the typical biohydrogen/VFAs-production trend.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (6.3)$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (6.4)$$

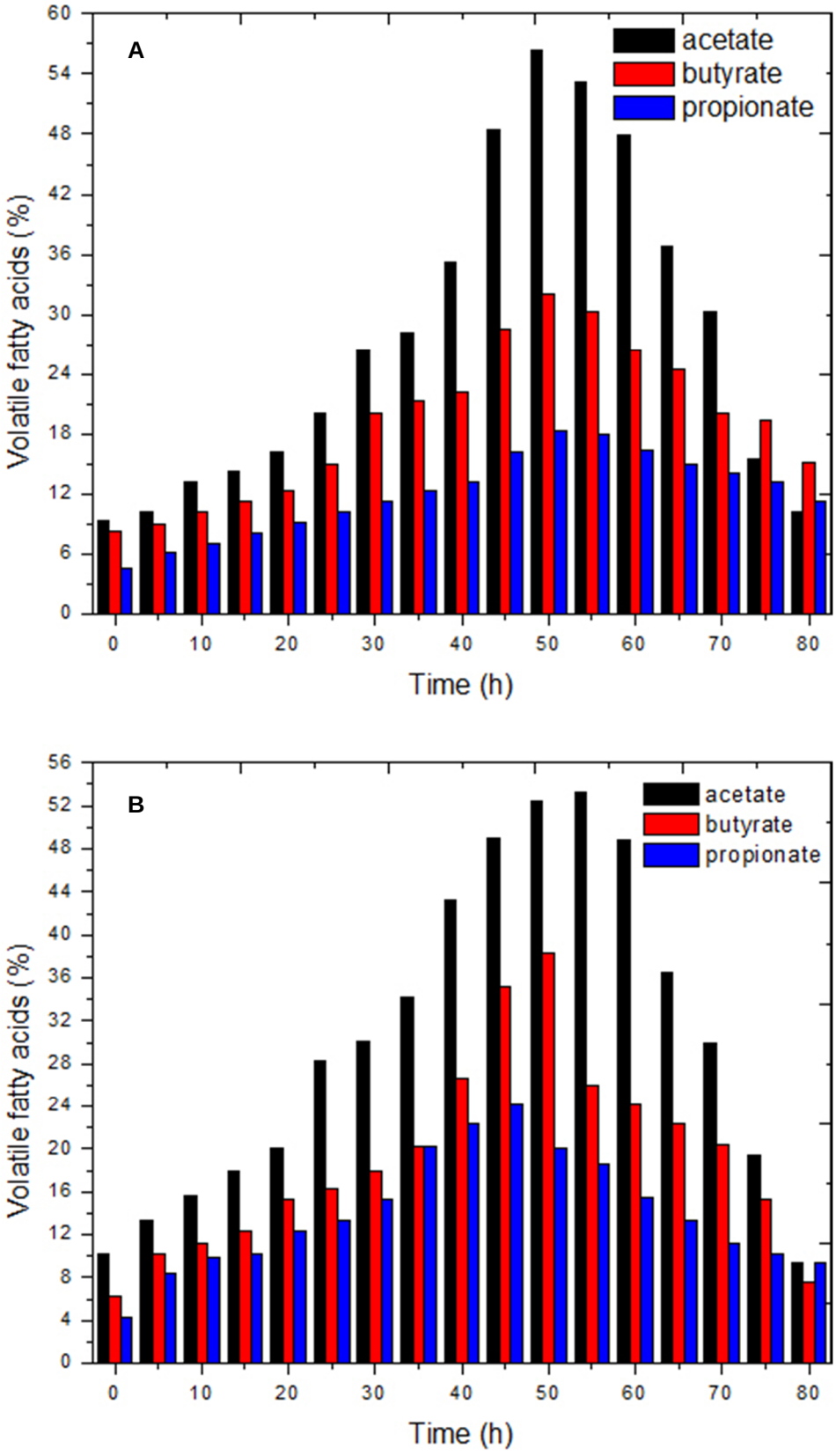


Figure 6.7: Volatile fatty acids production profile in biohydrogen production using (A) nitrogen gas sparged immobilized cells and nitrogen gas sparged suspended cells (B).

6.4 Summary

In this chapter, the effect of nitrogen gas sparging on dark fermentative biohydrogen production performance using suspended and immobilized cells of anaerobic mixed bacteria is reported. A maximum biohydrogen fraction of 56.98% with a concomitant biohydrogen production yield of 294.83 mL H_2/g TVS was achieved in the fermentation system using sparged encapsulated cells of anaerobic mixed sludge. The biohydrogen yield was 1.8 and 2.5 times higher than that of sparged and non-sparged suspended (control) cell system, respectively. The technique of nitrogen gas sparging and cell immobilization will play a pivotal role in optimizing and up-scaling the biohydrogen production process using solid biowaste feedstocks. These findings will be communicated to researchers via peer-reviewed journal publications.

References

APHA, (1998). Standard Methods for the Examination of Water and Wastewater. 20th Ed, Washington DC, USA, 125-186.

Bastidas-Oyanedel, J.R., Mohd-Zaki, Z., Zeng, R.J., Bernet, N., Pratt, S., Steyer, J.P., and Batstone, D.J. (2012). Gas controlled hydrogen fermentation. International Journal of Hydrogen Energy 110, 503-509.

Beckers, L., Hiligsman, S., Masset, J., Hamilton, C., and Thonart, P. (2012). Effects of hydrogen partial pressure on fermentative biohydrogen production by a chemotropic *Clostridium* bacterium in a new horizontal rotating cylinder reactor. Energy Procedia 29, 34-41.

Bru, K., Blazy, V., Joulian, C., Trably, E., Latrille, E., Quemeneur, M., and Dictor, M.C. (2012). Innovative CO_2 pretreatment for enhancing biohydrogen production from the organic fraction of municipal solid waste (OFMSW). International Journal of Hydrogen Energy 37, 14062-1471.

Bundhoo, M.A.Z., and Mohee, R. (2016). Inhibition of dark fermentative bio-hydrogen production: A review. International Journal of Hydrogen Energy 41, 6713-6733.

Chandrasekhar, K., Lee, Y.J., and Lee, D.W. (2015). Biohydrogen Production: Strategies to Improve Process Efficiency through Microbial Routes. International Journal of Molecular Science 16, 8266-8293.

Chong, M.L., Sabaratnam, V., Shirai, Y., and Hassan, M.A. (2009). Biohydrogen production from biomass and industrial wastes by dark fermentation. International Journal of Hydrogen Energy 34, 3277-3287.

Fan, Y.T., Zhang, Y.H., Zhang, S.F., Hou, H.W., and Ren, B.Z. (2006). Efficient conversion of wheat straw wastes into biohydrogen gas by cow dung compost. Bioresource Technology 7, 500-505.

Foglia, D., Wukovits, W., Friedl, A., De Vrije, T., and Claassen, P.A.M. (2011). Fermentative hydrogen production: influence of application of mesophilic and thermophilic bacteria on mass and energy balances. Chemical Engineering Transactions 25, 815-820.

Ghimire, A., Frunzo, L., Pirozzi, F., Trably, E., Escudie, R., Lens, P.N.L., and Esposito, G.A (2015). Review on dark fermentative biohydrogen production from organic biomass: Process parameters and use of by-products. Applied Energy 144, 73-95.

Guo, X.M., Trably, E., Latrille, E., Carre, H., and Steyer, J.P. (2010). Hydrogen production from agricultural wastes by dark fermentation: a review. International Journal of Hydrogen Energy 35, 10660-10673.

Jung, K.W., Kim, D.H., Kim, S.H., and Shin, H.S. (2011). Bioreactor design for continuous dark fermentative hydrogen production. Bioresource Technology 102, 8612-8620.

Karlsson, A., Vallin, L., and Ejlertsson, J. (2008). Effects of temperature, hydraulic retention time and hydrogen extraction rate on hydrogen production from the fermentation of food industry residues and manure. International Journal of Hydrogen Energy 33, 953–962.

Keskin, T., Giusti, L., and Azbar, N. (2002). Continuous biohydrogen production in immobilized biofilm system versus suspended cell culture. International Journal of Hydrogen Energy 37, 1418–1424.

Khanal, S.K., Chen, W.H., Li, L., and Sung, S. (2004). Biological hydrogen production: effects of pH and intermediate products. International Journal of Hydrogen Energy 29, 1123-1231.

Kim, D.H., Han, S.K., Kim, S.H., and Shin, H.S. (2006). Effect of gas sparging on continuous fermentative hydrogen production. International Journal of Hydrogen Energy 31, 2158–2169.

Kraemer, J.T., and Bagley, D.M. (2008). Optimisation and design of nitrogen-sparged fermentative hydrogen production bioreactors. International Journal of Hydrogen Energy 33, 6558–6565.

Kumar, G., Mudhoo, A., Sivagurunathan, P., Nagarajan, D., Ghimire, A., Lay, C.H., Lin, C.Y., Lee, D.J., and Chang, J.S. (2016). Recent insights into the cell immobilization technology applied for dark fermentative hydrogen production. Bioresource Technology 219, 725-737.

Levin, D.B., Pitt, L., and Love, M. (2004). Biohydrogen production: prospects and limitations to practical application. International Journal of Hydrogen Energy 29, 173-185.

Logan, B., Oh, S., Kim, I., and Van Ginkel, S. (2002). Biological hydrogen production measured in batch anaerobic respirometers. Environmental Science and Technology 36, 2530-2535.

Ma, Z., Li, C., and Su, H. (2017). Dark bio-hydrogen fermentation by an immobilized mixed culture of *Bacillus cereus* and *Brevumdimonas naejangsanensis*. Renewable Energy 105, 458-464.

Mandal, B., Nath, K., and Das, D. (2006). Improvement of biohydrogen production under decreased partial pressure of H_2 by *Enterobacter cloacae*. Biotechnology Letters 28, 831-835.

Mizuno, O., Dinsdale, R., Hawkes, F.R., Hawkes, D.L., and Noike, T. (2000). Enhancement of hydrogen production from glucose by nitrogen gas sparging. Bioresource Technology 73:56-65.

Mohanakrishna, G., Venkata Mohan, S., and Sarma, P.N. (2010). Utilizing acid-rich effluents of fermentative hydrogen production process as substrate for harnessing bioelectricity: An integrative approach. International Journal of Hydrogen Energy 35, 3440 – 3449.

Nguyen, T.A.D., Han, S.J., Kim, J.P., Kim, M.S., and Sim, S.J. (2010). Hydrogen production of the hyperthermophilic eubacterium, *Thermotoga neapolitana* under N_2 sparging condition. Bioresource Technology 101, S38-S41.

Nicolaou, S.A., Gaida, S.M., and Papoutsakis, E.T. (2010). A comparative view of metabolite and substrate stress and tolerance in microbial bioprocessing: from biofuels and chemicals, to biocatalysis and bioremediation. Metabolic Engineering 12, 307-331.

Oh, S.E., Zuo, Y., Zhang, H., Guiltinan, M.J., Logan, B.E., and Regan, J.M. (2009). Hydrogen production by *Clostridium acetobutylicum* ATCC 824 and megaplasmid-deficient mutant M5 evaluated using a large headspace volume technique. International Journal of Hydrogen Energy 34, 9347-9353.

Pachapur, V.L., Sarma, J.S., Brar, S.K., Bihan, Y.L., Buelna, G., and Soccol, C.R. (2015). Evidence of metabolic shift on hydrogen, ethanol and 1,3-propanediol production from crude glycerol by nitrogen sparging under micro-aerobic conditions using co-culture of *Enterobacter aerogenes* and *Clostridium butyricum*. International Journal of Hydrogen Energy 40, 8669-8676.

Penniston, J., and Gueguim Kana, E.B. (2016). Impact of Culture pH Regulation on Biohydrogen Production using Suspended and Immobilized Microbial Cells. Preprints, 1-21.

Sekoai, P.T., Awosusi, A.A., Yoro, K.O., Singo, M., Oloye, O., Ayeni, A.O., Bodunrin, M., and Daramola, M.O. (2017). Microbial cell immobilization in biohydrogen production: a short overview. Critical Reviews in Biotechnology, 1-15.

Srikanth, S., and Venkata Mohan, S. (2012). Regulatory function of divalent cations in controlling the acidogenic production process. RSC Advances 2, 6576-6589.

Tanisho, S., Kuromoto, M., and Kadokura, N. (1998). Effect of CO_2 removal on hydrogen production by fermentation. International Journal of Hydrogen Energy 23, 559–563.

Van Ginkel, S.W., Lay, J.J., and Sung, S. (2001). Biohydrogen production as a function of pH and substrate concentration. Environmental Science and Technology 35, 4726-4730.

Veeravalli, S.S., Chaganti, S.R., Lalman, J.A., and Heath, D.D. (2014). Fermentative H_2 production using a switchgrass steam exploded liquor fed to mixed anaerobic cultures: Effect of hydraulic retention time, linoleic acid and nitrogen sparging. International Journal of Hydrogen Energy 39, 9994-10002.

Venkata Mohan, S. (2009). Harnessing of biohydrogen from wastewater treatment using mixed fermentative consortia: Process evaluation towards optimization. International Journal of Hydrogen Energy 34, 7460-7474.

Xiao, L., Deng, Z., Fung, K.Y., and Ng, K.M. (2013). Biohydrogen generation from anaerobic digestion of food waste. International Journal of Hydrogen Energy 38, 13907-13913.

Yasin, H.M.Y., Mumtaz, T., Hassan, M.A., and Rahman, N.A.A. (2013). Food waste and food processing waste for biohydrogen production: A review. Journal of Environmental Management 130, 375-385.

Yerushalmi, L., Volesky, B., and Szczesny, T. (1985). Effect of increased hydrogen partial pressure on the acetone-butanol fermentation by *Clostridium acetobutylicum*. Applied Microbiology and Biotechnology 22, 103-107.

Chapter 7–Conclusions and Recommendations for future work

7.1 Summary and Conclusions

South Africa is one of the countries in the world whose important components of government policies have focused on energy efficiency and energy savings. Consequently, several policies are being made in response to a range of challenges, which include high-energy prices, security of energy supply, and environmental protection. Some of the proposed strategies include the implementation of clean and sustainable energy resources. Dark fermentative biohydrogen production holds a huge potential as a future energy resource in South Africa and the rest of the world. However, two major barriers must be first addressed in order to realize its industrial application. These include low process yields and high substrate costs which account for almost 60% of the overall costs. Therefore, this research focused on the utilization of South African solid biowaste materials to demonstrate the economic viability of biohydrogen production from these feedstocks. The research also explored various biohydrogen optimization techniques to maximize its yields. These are the major highlights:

- The feasibility of dark fermentative biohydrogen production using South African agricultural, municipal, and industrial waste materials was demonstrated in this research. It was observed that feedstocks rich in carbohydrates are suitable for dark fermentation process due to their biodegradable nature and high biohydrogen production potential. A maximum biohydrogen fraction of 43.98, 40.32 and 38.12% with a corresponding yield of 278.36, 238.32 and 215.69 mL H_2/g TVS was obtained from potato, cabbage, and brewery waste, respectively. Therefore, utilization of solid biowaste materials is one of the most effective ways of overcoming some of the economic constraints in biohydrogen process development because they are highly accessible, rich in nutritional composition, and considered waste. Furthermore, waste

beneficiation approaches through dark fermentative biohydrogen production will significantly assist to mitigate environmental pollution while generating clean and sustainable energy sources in South Africa.

- The microbial composition in the fermentation broth of a suitable substrate (potato waste) was investigated using PCR-based 16S rRNA technique. The study indicated the dominance of *Clostridium* species which are the active microorganisms that participate in biohydrogen production during the dark fermentation process.
- Response surface methodology (RSM) approach via central composite design (CCD) was used in this research to model and optimize the operating variables such as pH, temperature, fermentation time, and substrate concentration during biohydrogen production using potato waste. A 2^4-CCD using STATISTICA 8 release 7 Statistical Software was employed to generate 26 duplicated batch fermentation experiments which were carried out to improve biohydrogen yield. The optimized operating variables were 39.56 g/L, 5.56, 37.87 ^oC, and 82.58 h for potato waste concentration, pH, temperature, and fermentation time, respectively. These values resulted to a biohydrogen yield of 68.54 mL H_2/g TVS. The optimization study produced a biohydrogen yield of 79.43 mL H_2/g TVS which was 15.9% higher than the model predicted yield. These results highlight the importance of optimization tools in biohydrogen process development and they could be instrumental towards fast-tracking the commercialization of the process.
- The industrialization of any bioprocess relies on its scalability. Therefore, the operating conditions were evaluated in a biohydrogen scale-up study using cell immobilization technology of calcium alginate beads as inoculum. Biohydrogen scale-up study was conducted in a 13 L Benchtop INFORS HT reactor. The system produced a peak biohydrogen fraction of 56.38% and biohydrogen yield of 298.11 mL

H_2/g TVS. The performance of this scale-up system was superior to scale-up study that employed suspended cultures as shown in chapter 4. Therefore, utilization of immobilized biocatalysts could pave a way for large-scale biohydrogen production, and could help to overcome some of the pressing challenges such as accumulation of oxygen in the reactor headspace, rapid drop in pH, contamination, and inconsistent mixing faced by this process.

- Metal ions play a crucial role in the metabolism of biohydrogen-producing pathways because they affect the hydrogenase enzymes, substrate uptake, and biohydrogen yield. These supplementary nutrients were also evaluated for dark fermentative biohydrogen production using suspended and immobilized cells. A maximum biohydrogen fraction of 45.21% and process yield of 292.8 mL H_2/g TVS was obtained in batch experiment using Fe^{2+} (1000 mg/L) and immobilized cells. The value was 1.3 times higher than that of suspended culture system.
- The partial pressure is another parameter that cannot be overlooked in biohydrogen process development because it increases during the fermentation process and minimizes the overall yields. Therefore, it should be controlled during the acidogenic process to maximize the biohydrogen conversion efficiency. Gas sparging is one of the most effective methods of reducing the partial pressure in the liquid phase. In this work, a batch system using nitrogen gas sparging and immobilized cells enhanced the biohydrogen production efficiency. A high biohydrogen fraction of 56.98%, which corresponded to a biohydrogen yield of 294.83 mL H_2/g TVS, was obtained. The yield was 1.8 and 2.5 times higher than that of the nitrogen gas sparged and non-sparged (control) system, respectively. Nitrogen gas sparging coupled with cell immobilization is a novel approach that could be used to surpass the low biohydrogen production yields and could ultimately lead to scalability of the process.

7.2 Recommendations for future work

Commercialization of biohydrogen process technology will assist in the intensification of clean and sustainable energy resources, and the mitigation of environmental pollution. Nevertheless, there are many technical challenges that must be overcome before the commercialization of a biohydrogen driven economy. These recommendations are proposed for future research:

- More biohydrogen production studies should be conducted at pilot-scale using novel bioreactor configurations incorporated with various online-monitoring and regulating devices (e.g. pH sensors, actuators, dissolved oxygen sensors, etc). This will provide reliable fermentation data that can be used for its large-scale production.
- Inoculum development still remains a critical issue in biohydrogen process development. Research relating to metabolic engineering and cell immobilization will help in: the creation of oxygen tolerant microbial cells, inhibition of biohydrogen-consuming pathways, reducing the levels of contamination, enabling the reusability of cells, extending the fermentation periods, maintaining anaerobic conditions, and increasing the biohydrogen yields.
- Integrated processes should be implemented in dark fermentation to enhance the energy yields and substrate conversion efficiency. Dark fermentation can be integrated with microbial electrolysis cells, photo-fermentation processes, microbial fuel cells, and biomethane production. However, the process costs need to be taken into account when using these systems especially at large-scale.
- More research should also focus on biohydrogen purification systems and biohydrogen storage systems to accelerate the development of this technology.

APPENDIX A

Copies of publications